PATENT
Docket No. 293.00010102

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Daryll A. EMERY et al.	Group Art Unit:	1655
Serial No.:	10/749,602	Examiner:	Patricia A. Leith
Confirmation No.:	8548		
Filed:	December 31, 2003		
For:	IN OVO DELIVERY OF AN IMMUNOGEN CONTAINING IMPLANT		

APPEAL BRIEF

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Commissioner for Patents
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Sir:

This Brief is presented in support of the Appeal filed October 15, 2009, from the final rejection of claims 34-44, 67-69, 71-82 and 84-102 of the above identified application under 37 C.F.R. §§1.113 and 1.191.

This Brief is being submitted as set forth in 37 C.F.R. §41.37. Please charge Deposit Account No. 13-4895 the fee for filing this Brief under 37 C.F.R. §41.20(b)(2).

I. REAL PARTY IN INTEREST

The real party in interest of the above-identified patent application is the assignee, Epitopix, LLC.

II. RELATED APPEALS AND INTERFERENCES

There are no appeals or interferences known to Appellant's Representatives which would directly affect, be directly affected by, or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 34-44, 67-69, 71-82, and 84-102 are pending and are the subject of this Appeal (see Claim Appendix).

Claims 34-44, 67-69, 71-82, and 84-102 stand rejected.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claim 34 is drawn to a method for inducing adaptive immunity in a bird against a selected immunogen. The method includes injecting a biocompatible implant into an egg, wherein the biocompatible implant comprises the selected immunogen and a biocompatible matrix material, wherein the egg comprises maternal antibody to the selected immunogen, wherein the implant provides for sustained release of the immunogen until the maternal antibodies in a bird hatching from the egg are reduced so that the bird is capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a gram-negative bacterium.

In ovo injection of an implant is described at, for example, page 9, lines 9-20. The biocompatible implant and biocompatible matrix material are described at, for example, from page 10, line 21 through page 14, line 16. Sustained release of the immunogen from the implant is described at, for example, page 10, lines 4-9. Siderophore receptor protein immunogens are described at, for example, from page 19, line 18 through page 20, line 10. Administering the biocompatible implant into an egg that includes maternal antibodies to the selected immunogen – i.e., when the egg is immuno-incompetent to mount an active immune response against the selected immunogen – is described at, for example, from page 7, line 19 through page 9, line 5.

Claim 69 is drawn to a method for inducing adaptive immunity in a bird against a selected immunogen. The method includes injecting a biocompatible implant *in ovo*, wherein the biocompatible implant comprises the selected immunogen and a biocompatible matrix material, and hatching eggs to result in birds, wherein the eggs comprise maternal antibody to the immunogen, wherein the implant provides for sustained release of the immunogen until a time when maternal antibodies of the birds to the immunogen are sufficiently reduced so that the birds are capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a bacterium.

In ovo injection of an implant is described at, for example, page 9, lines 9-20. The biocompatible implant and biocompatible matrix material are described at, for example, from page 10, line 21 through page 14, line 16. Sustained release of the immunogen from the implant is described at, for example, page 10, lines 4-9. Siderophore receptor protein immunogens are described at, for example, from page 19, line 18 through page 20, line 10. Hatching the eggs as part of the method is described at, for example, from page 8, line 4 through page 9, line 5. Administering the biocompatible implant into an egg when maternal antibodies of the birds to the immunogen are sufficiently reduced so that the birds are capable of mounting an adaptive immune response to the immunogen is described at, for example, from page 7, line 19 through page 8, line 11.

Claim 84 is drawn to a method for inducing adaptive immunity in a population of birds against a selected immunogen. The method includes injecting a biocompatible implant into a population of eggs that comprise maternal antibody to the selected immunogen, wherein the biocompatible implant comprises the selected immunogen and a biocompatible matrix material, wherein the implant provides for sustained release of the immunogen until the maternal antibodies to the immunogen in birds hatching from the eggs are reduced, and the birds hatched from the eggs are capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a bacterium.

In ovo injection of an implant is described at, for example, page 9, lines 9-20. Biocompatible implants are described at, for example, from page 10, line 21 through page 11,

line 10 and page 14, lines 6-16. Administering the biocompatible implant to the eggs at a time when the eggs comprise maternal antibody to the selected immunogen is described at, for example, from page 7, line 19 through page 9, line 5. Siderophore receptor protein immunogens are described at, for example, from page 19, line 18 through page 20, line 10. Biocompatible matrices are described at, for example, from page 10, line 21 through page 13, line 19. Sustained release of the immunogen from the implant and until the maternal antibodies to the immunogen in birds hatching from the eggs are reduced is described at, for example, page 10, lines 4-9 and from page 7, line 19 through page 9, line 5.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Whether claims 34, 37, 39-43, 67-69, 84-86, 89, 91-95, and 97-102 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,538,733), in view of Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766).

The Final Rejection further identifies claim 83 as rejected under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,538,733), in view of Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766). However, claim 83 was canceled in Appellants' response filed May 15, 2007 and is, therefore, not subject to the instant Appeal.

Whether claims 34-44, 67-69, 71-82 and 84-102 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766) in view of Emery et al. (U.S. Patent No. 5,538,733), and further in view of Evans et al. (U.S. Patent No. 6,500,438).

VII. ARGUMENT

Claims 34, 37, 39-43, 67-69, 84-86, 89, 91-95, and 97-102 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,538,733), in view of Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766).

Claims 34-44, 67-69, 71-82 and 84-102 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766) in view of Emery et al. (U.S. Patent No. 5,538,733), and further in view of Evans et al. (U.S. Patent No. 6,500,438).

Appellants respectfully submit that the Examiner has erred in maintaining the rejections that are the subject of this Appeal and respectfully request that the rejections be reversed.

35 U.S.C. §103(a) states, "A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made."

In *KSR International Co. v. Teleflex Inc.* (127 S.Ct. 1727, 167 L.Ed.2d 705, 550 U.S. 398, 82 USPQ2d 1385 (U.S. 2007)), the United States Supreme Court addressed the circumstances under which a patent claim is obvious under 35 U.S.C. §103(a). After reviewing Court precedent, the Court summarized as follows:

> The principles underlying these cases are instructive when the question is whether a patent claiming the combination of elements of prior art is obvious. ... If a person of ordinary skill can implement a predictable variation, §103 likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill. *Sakraida* and *Anderson's-Black Rock* are illustrative—a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions. 82 USPQ2d, 1396. (emphasis added).

Appellants respectfully submit that a person skilled in the art could not have predictably implemented the variations to the '733 patent that the Final Office Action suggests is

taught by the '479 patent and '766 patent and arrived at the subject matter of claims 34, 37, 39-43, 67-69, 84-86, 89, 91-95, and 97-102. Similarly, Appellants respectfully submit that one skilled in the art could not have predictably implemented the variations to the '479 patent that the Final Office Action suggests is taught by the '733 patent, '766 patent, and the '438 patent and arrived at the subject matter of claims 34-44, 67-69, 71-82 and 84-102.

A. Claims 34, 37, 39-43, 67-69, 84-86, 89, 91-95, and 97-102 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,538,733), in view of Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766)

1. Claims 34, 37, 39-43, 67, 68, 97, and 100 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,538,733), in view of Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766).

Claim 34 is independent. Each of claims 37, 39-43, 67, 68, 97, and 100 depends, directly or indirectly, from claim 34 and, therefore, includes all of the features recited in claim 34. Thus, remarks that refer to claim 34 apply equally to each of 37, 39-43, 67, 68, 97, and 100.

Claim 34 recites a method for inducing adaptive immunity in a bird against a selected immunogen. Generally, the method includes injecting a biocompatible implant into an egg, wherein the biocompatible implant comprises the selected immunogen and a biocompatible matrix material, wherein the egg comprises maternal antibody to the selected immunogen, wherein the implant provides for sustained release of the immunogen until the maternal antibodies in a bird hatching from the egg are reduced so that the bird is capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a gram-negative bacterium.

Appellants respectfully submit that the Examiner errs in concluding that a person skilled in that art could have predictably implemented the variations to the '733 patent that the Final Office Action suggests is taught by the '479 patent and '766 patent and arrived at the subject matter of claim 34.

The Examiner asserts that the '733 patent discusses the problem of vaccinating young animals because maternal antibodies are present in neonates and may interfere with the young animal's ability to generate an immune response against an antigen in the vaccine. The Examiner asserts that the '733 patent proposes, as a solution, administering a vaccine via a sustained and delayed release delivery agent between the post-hatch age of 1 and 90 days. (Final Office Action, page 4). The Examiner also asserts that the '733 patent teaches siderophore receptor proteins (SRPs) from gram negative bacteria as preferred immunogens and notes that the '733 patent expressly teaches delivering the SRPs to the animal at a post-hatch age of between 1 and 60 days. (Final Office Action, page 5).

The Examiner acknowledges that the '733 patent fails to teach administering the SRP by sustained release *in ovo*. (Final Office Action, page 6). The Examiner continues to acknowledge other asserted deficiencies in the '733 patent. However, these acknowledgments are not directly relevant to the subject matter of claim 34 and indicate at least one area in which the Examiner misunderstands an important feature of the method of claim 34. Appellants will address these points after addressing the fundamental weakness of the rejection.

The Examiner asserts that the '479 patent teaches immunizing poultry with an SRP from a gram negative bacterium, that the SRP can be administered *in ovo*, and that the SRP can be delivered by sustained release. (Final Office Action, page 6-7).

The Examiner asserts that the '766 patent discloses a method for introducing material into poultry eggs. (Final Office Action, page 7).

The Examiner concludes that one of ordinary skill in the art at the time the invention was made would have been motivated to administer a sustained release formulation including an SRP *in ovo*. (Final Office Action, page 8). Appellants respectfully disagree.

In support of her conclusion, the Examiner cites the U.S. Supreme Court in *KSR International Co. v. Teleflex Inc.* as follows, "The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results." 82 USPQ2d, 1395.

Appellants do not dispute that the method of claim 34 involves features that, separately, were previously known in the art. However, the Examiner errs in failing to recognize that the method recited in claim 34 reflects more than simply combining familiar elements according to known methods to yield predictable results. Because the method recited in claim 34 reflects more than simply combining familiar elements according to known methods to yield predictable results, Appellants submit that claim 34 is patentable over the combination of the '733 patent, '479 patent, and the '766 patent.

Appellants submit that prior to Appellants disclosure, one skilled in the art could not have combined the teaching of the '733 patent, '479 patent, and the '766 patent as suggested by the Examiner and predictably arrived at the method of claim 34. In particular, the portion of the '479 patent that the Examiner asserts teaches delivery of SRPs *in ovo* does not account for the presence of maternal antibodies against the selected immunogen in the unhatched egg. Moreover, one skilled in the art would have understood that the immunological environment of the newly hatched bird and a soon-to-be-hatched egg are different enough that the skilled person could not predictably extrapolate the teaching of the '733 patent to *in ovo* administration of the sustained release implant.

The method of claim 34 allows those in the poultry industry to vaccinate a generation of birds while the birds are relatively easy to handle – i.e., prior to hatching. Thus, one can protect an entire generation of birds while administering vaccinations to each individual at one time. The desire to protect a generation of eggs in this way faces at least two significant challenges, each of which is explained in Appellants' specification.

First, in the first few weeks of life a newborn chick, poult, duckling or other avian hatchling (hereinafter, collectively "chick" for brevity) is relatively incompetent at producing antibodies in response to antigenic stimuli. During this period, a significant amount of resistance to infectious diseases is provided by passive immunity derived from maternal antibodies of the hen. Passive immunity can be transferred from the hen to the chick *in ovo*. IgY antibodies are sequestered from the hen's serum, secreted in the ovary, and incorporated into the yolk before

ovulation. Of course, only IgY antibodies against immunogens to which the hen has been exposed recently enough to still have circulating IgY antibodies in her serum can be sequestered and secreted to the ovary. Thus, with respect to a specific immunogen – e.g., a selected SRP from a certain gram negative bacterium – while one hen may have been exposed to the immunogen recently enough so that her circulating IgY against the immunogen is at its peak, another hen may have been exposed so recently (e.g., less than eight days) so that circulating IgY against the immunogen is nonexistent or less than full strength, while another hen may have been exposed to the immunogen so long ago (e.g., more than six weeks) that circulating IgY against the immunogen has waned, while yet another hen may never have been exposed to the immunogen and, therefore, have no circulating IgY against the immunogen. One important implication of this is that the transfer of maternal antibodies to eggs is not consistent across hens of a flock – i.e., each hen may differ in not only the qualitative ability to provide maternally-derived antibodies against a selected immunogen, but also the quantity of maternally-derived antibody against a selected immunogen. Thus, within any given population of egg-laying hens, one expects to find varying levels of serum antibody raised against any particular immunogen. This variation within the flock is passed to the eggs, which results in variation of the amount of maternal antibody against any particular immunogen among the eggs of a given generation – variation that is directly correlated with the varying amounts of serum antibody against the particular immunogen present in the flock of egg-laying hens at the time that the hens lay eggs.

Maternally-derived antibodies are stored in the yolk until the later stages of embryonic development when they are absorbed by the embryonic membranes and transferred to the circulation of the chick to provide passive immunity. Maternally-derived antibodies provide immunological protection of the newly hatched chick during the period before the chick's own immune system can actively produce antibody against immunogens of pathogens. The amount of maternally-derived antibody against a selected immunogen influences the duration of passive immunity conferred to the newly hatched chick.

However, the presence of maternally-derived antibodies can also interfere with the ability of the young bird to actively respond to an immunogen and, instead, induce immune

tolerance. Immune tolerance to a foreign antigen can occur when a subject is exposed to a foreign antigen under conditions that elicit specific unresponsiveness to the foreign antigen rather than an adaptive humoral immune response to the antigen. In other words, under some circumstances, exposure to a foreign antigen does not necessarily result in the challenged subject mounting an adaptive immune response, but instead results in the subject's immune system perceiving the foreign antigen as "self" and establishing antigen-specific immune non-response.

In summary, prior to Appellants' invention, those in the poultry industry wanting to vaccinate a new generation of birds must weigh the following challenge. To ensure that all of the chicks are vaccinated at a time when the chicks are able to mount an adaptive immune response to immunogens in the vaccine, one must wait until (a) each chick's immune system is competent to raise an adaptive immune response to the immunogens in the vaccine, and (b) maternally-derived antibodies to the selected immunogens have waned so as not to interfere with the ability of the chick's immune system to recognize the vaccine immunogens. However, this option leaves certain chicks – those chicks without maternally-derived passive immunity to the particular immunogen – unprotected and at risk to infection because (a) the qualitative existence of, and (b) the amount of maternally-derived antibodies against a particular immunogen varies from egg to egg because the fact, extent, and timing of exposure of the hen to a pathogen harboring the particular immunogen varies from hen to hen.

The '733 patent suggests providing the chick with a sustained release implant that releases immunogens over time so that the immunogen will be present when (a) the chick's immune system is competent to raise an adaptive immune response and (b) maternally-derived antibodies to the selected immunogens have waned so that the chick's immune system can take over. The Examiner errs in concluding that the method of claim 34 represents little more than administering the sustained release implant of the '733 patent *in ovo* rather than post-hatch. So why wouldn't the skilled person modify the teaching of the '733 patent to administer the implant *in ovo*? After all, eggs are easier to handle than even one-day old chicks and methods for introducing materials into eggs were known as of the filing date of the '733 patent. M.P.E.P. §2145(X)(D) hints at the answer, stating:

> The totality of the prior art must be considered, and proceeding contrary to accepted wisdom in the art is evidence of nonobviousness. *In re Hedges*, 783 F.2d 1038, 228 USPQ 685 (Fed. Cir. 1986) (Applicant's claimed process for sulfonating diphenyl sulfone at a temperature above 127°C was contrary to accepted wisdom because the prior art as a whole suggested using lower temperatures for optimum results as evidenced by charring, decomposition, or reduced yields at higher temperatures.).
>
> Furthermore, "[k]nown disadvantages in old devices which would naturally discourage search for new inventions may be taken into account in determining obviousness." *United States v. Adams*, 383 U.S. 39, 52, 148 USPQ 479, 484 (1966).

Additionally, the U.S. Supreme Court in *KSR* approvingly referred to the Court's *U.S. v. Adams* decision as follows: "The Court relied upon the corollary principle that when a prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious." 82 USPQ2d, 1395 (emphasis added).

Simply put, the immunological environment of the unhatched embryo is known to those skilled in the art to be sufficiently different from the one-day old chick that (a) one could not predict that sustained release of immunogen *in ovo* when maternally-derived antibodies to the immunogen are present would induce a protective adaptive immune response in the chick once hatched, and (b) the prior art teaches away from sustained release of the immunogen *in ovo* when maternally-derived antibodies to the immunogen are present because of the risk of inducing exactly the opposite response: immune tolerance.

The issue in the Appeal is not whether it was technically possible to administer a sustained release implant *in ovo*. The issue is whether the skilled person would have done so and been able to predict that an adaptive immune response would result. Appellants submit that the skilled person could not predict that an adaptive immune response against immunogens in the implant would result. Moreover, the prior art teaches the skilled person that sustained release vaccination *in ovo* when maternally-derived antibodies against the immunogen are present may induce immune tolerance.

Many of the conditions under which immune tolerance may be induced are present in the circumstance of *in ovo* vaccination as recited in claim 34. (See, *Microbiology*,

fourth edition, Davis *et al.* eds., 1990, J.B. Lippincott Co., Philadelphia, Pennsylvania, pp. 381-382.). For example, the selected immunogens, SRPs, are monomeric antigens, not aggregated; sustained release implants are more similar to intravenous administration than injection into tissue; and *in ovo* administration necessarily results in vaccination of the embryo rather than adult. So, one skilled in the art would recognize that vaccinating embryos using sustained release of SRPs harbors the risk of inducing immune tolerance to the SRPs in the vaccine rather than raising adaptive immunity against the SRPs. Many of these conditions are present whether the sustained release implant is administered at one day of age (as in the '733 patent) or *in ovo*, as in the present claims.

The difference between vaccinating an egg by sustained release of a selected immunogen from a biocompatible implant at one day of age versus injecting the biocompatible implant *in ovo* – and a compelling reason why one skilled in the art would not extend the teaching of the '733 patent to *in ovo* delivery – is that the risk of inducing immune tolerance to the immunogen is greater when the biocompatible implant containing the immunogen is delivered *in ovo* compared to the biocompatible implant being delivered after hatch. In other words, the multifactorial risk of inducing the exact opposite of the intended immunological response is magnified when the sustained release implant is administered *in ovo* rather than after hatching. One reason for the increased risk of inducing immune tolerance when the biocompatible implant is injected *in ovo* is the different amounts of – and the corresponding effects of the different amounts of – circulating maternal antibody in the embryo versus in the newly-hatched chick.

Claim 34 recites that the egg into which the biocompatible implant is injected comprises maternal antibody to the selected immunogen. Recall that across a generation of eggs, the qualitative presence of maternally-derived antibodies against a selected immunogen and the quantitative amount of maternally-derived antibodies against a selected immunogen vary from egg to egg. Prior to hatch, some of the maternal antibodies circulate in the embryo but most remain sequestered in the yolk. At hatching, however, the yolk is fully absorbed and the maternal antibodies from the yolk are fully absorbed into the circulation of the chick. Thus, the

chick – but not the embryo – has the full passive immunization benefit of the maternal antibodies, which may or may not include maternal antibodies against a particular immunogen. Consequently, the circulating maternal antibody environment is very different in the embryo than in the newly-hatched chick and this difference influences the risk of inducing immune tolerance.

The difference in the circulating maternal antibody environments present in the embryo and the newly-hatched chick dictate the different immunological responses that one skilled in the art would have expected to vaccines administered by sustained release implant *in ovo* versus post-hatch. As just explained, a day-old chick possesses the full amount of maternal antibody in circulation, including any maternal antibody specific to the selected immunogen. When a day-old chick is vaccinated with a biocompatible implant providing sustained release of the selected immunogen, maternal antibodies against the selected immunogen – if present at all – clear the immunogen from the chick's circulation without involving the chick's immature immune system, thereby reducing the risk of inducing immune tolerance to the selected immunogen. In contrast, when a biocompatible implant is provided *in ovo*, the level of maternal antibody absorbed by the embryo – e.g., at day 20 of incubation as described in Example 4 – is incomplete and, as a consequence, the immunogen is less likely to be cleared by maternal antibodies against the immunogen. As a result, the embryo is more likely to be exposed to the immunogen. Because of the immaturity of the embryo's immune system, the embryo's exposure to the immunogen presents the risk that the embryo will recognize the immunogen as "self" rather than "foreign" and, as a consequence, the embryo is at risk for developing immune tolerance to the immunogen in the biocompatible implant rather than adaptive immunity against the immunogen.

At least two factors put the embryo at greater risk for inducing immune tolerance to the selected immunogen than a newly-hatched chick receiving the very same sustained release implant. First, the embryo's immune system is less mature and, therefore, is less capable of raising an adaptive response to a foreign antigen and is, therefore, more susceptible to inducing immune tolerance to the foreign antigen. Second, the immune system of an embryo is less protected from the foreign antigen by maternal antibodies, if present at all, than the immune system of a day-old chick. Each factor, alone, is sufficient to render the effect of administering

the sustained release implant to an embryo unpredictable. Taken together, however, one skilled in the art could not have predicted that vaccinating eggs using the recited implant would provide effective vaccination rather than inducing immune tolerance.

Thus, prior to Appellants' disclosure, it was unpredictable whether injecting a biocompatible implant containing a selected immunogen into an egg that possesses maternal antibody against the selected immunogen could induce an adaptive immune response against a selected immunogen or, alternatively, whether doing so would induce immune tolerance to the selected immunogen.

Because the combination of the '733 patent and the '479 patent fails to provide one skilled in the art with a reasonable expectation that injecting a biocompatible implant containing a selected immunogen *in ovo* would induce an effective adaptive immune response rather than inducing immune tolerance, the combination does not help establish a *prima facie* case of obviousness against claim 34. Nothing in the '766 patent cures this deficiency in the combined teachings of the '733 patent and the '479 patent.

The Examiner errs in focusing on whether SRPs were known to be immunogens (yes, see, e.g., the '479 patent), whether sustained release of SRPs was known (yes, see, e.g., the '733 patent), and whether implanting materials *in ovo* was possible (yes, see, e.g., the '766 patent). The Examiner errs by ignoring – and what one skilled in the art was unable to predict prior to Appellants' disclosure – the biological consequence of providing a sustained release implant *in ovo* to eggs that contain maternally-derived antibodies to an immunogen released by the implant.

In responding to Appellants' arguments in the Final Office Action (pages 12-21), the Examiner errs by confusing the teachings provided in the cited documents. The Examiner states, "[T]he prior art already recognizes the use of SRPs for vaccination into poults as well as in-vivo [sic] via use of sustained delivery matrices." (Final Office Action, page 13). The prior art fails to recognize the use of SRPs for vaccination *in ovo* using sustained delivery matrices

implanted into eggs having maternally-derived antibodies to an immunogen released by the implant.

The Examiner errs in her interpretation of the '479 and '733 patents, stating, "The only teaching lacking between the Emery et al. patents [the '479 patent and the '733 patent] and the claimed invention is the age of the egg at vaccination. (Final Office Action, page 15). This statement is incorrect. A second difference between the teaching of the Emery et al. patents and the method of claim 34 is the presence of maternally-derived antibody to the SRP. Moreover, the difference highlighted in the statement is precisely the difference – i.e, between unhatched embryo versus day-old chick – that is responsible for the different immunological environments that the Examiner fails to recognize as creating the unpredictability recognized in the prior art.

The Examiner further errs, stating, "Although neither Emery et al. patents [sic] explicitly demonstrated *in-ovo* [sic] vaccination of SRP proteins at the age of the egg as indicated by claims 35 and 36 for example, determining a time to vaccinate poultry eggs with known vaccines such as SRPs which were already known to be delivered in delayed/sustained release matrices at the times as required by the claims is deemed well-within [sic] the skill level of the ordinary artisan and would have been achieved through routine optimization/experimentation." (Final Office Action, page 16). While this may be true for methods that involve vaccinating eggs that lack maternally-derived antibodies to the SRPs, it is not true in the case of vaccinating eggs having maternally-derived antibodies against the SRPs because of the risk of inducing immune tolerance rather than a protective adaptive immune response.

The Examiner further errs and displays confusion about the nature of Appellants' invention, stating, "The Specification as a whole appears to be solving an asserted problem of delivering an SRP protein to a young poult in such a manner as to deliver said SRP at a time when maternal antibodies are reduced." This is an incomplete understanding of not only the actual – as opposed to asserted – problem faced by those doing business in the poultry industry, but also the solution presented by Appellants. The problem further involves how to vaccinate literally thousands of animals efficiently – in both the immunological and economical sense – in

the very real commercial circumstances in which the qualitative and quantitative extent to which each egg has maternally-derived antibodies against a selected immunogen such as, for example, an SRP (a) differs and (b) cannot readily be determined. Seeking immunological efficiency suggests delaying vaccination until maternal antibodies in the chicks wane and can no longer interfere with the chicks' ability to generate an adaptive immune response at the economical expense of knowing that some chicks will be susceptible to infection. Seeking economical efficiency suggests vaccinating the animals as few times as necessary and while the vaccinees are easiest to handle. However, doing so risks (a) interference by maternal antibodies with the induction of an adaptive immune response to the vaccine and (b) the induction of immune tolerance against the selected immunogen.

Appellants' solution permits vaccination at a single time with a sustained release implant so that each vaccinee can receive the same vaccination and the chick will be immunologically protected regardless of when maternal antibodies against the SRP – if present at all – wanes. To this extent, Appellants' solution is similar to that described in the '733 patent. Appellants' solution goes further, however. Appellants' solution involves doing so prior to hatching, when the unhatched eggs are easier to handle than hatched chicks and in the face of the risk of inducing immune tolerance created by the combination of exposing the embryo *in ovo* to immunogens against which the embryo carries maternally-derived antibodies.

The Examiner asserts that "[t]he prior art already recognized that the presence of maternal antibodies in young poultry hastened the need for delivery of vaccines at a time when maternal antibodies in the poultry were reduced." (Final Office action, pages 17-18). Once again, this statement oversimplifies the problem facing those in the poultry industry and the solution provided by Appellants. Appellants do not dispute that the prior art described a need to provide immunological protection to young poultry when maternal antibodies against a selected immunogen are reduced. However, the prior art fails to recognize that one could do so by vaccinating *in ovo* even in the presence of maternal antibodies against the selected immunogen, when the immunological developmental stage of the embryo creates a level of risk of inducing

immune tolerance that is greater than when the very same vaccine is used to vaccinate hatched chicks.

Thus, the method recited in claim 34 is more than the combination of familiar elements according to known methods to yield no more than predictable results. Indeed, the method of claim 34 combines certain known elements in a way that the prior art suggests could lead to precisely the opposite result – i.e., immune tolerance to the selected immunogen.

As explained above, the biological and immunological reality is more complex than simply moving the time of providing the implant ahead by two days. To reiterate: because of the difference in the immunological environment of an unhatched embryo and a one-day old hatched chick, it was unpredictable whether providing a sustained release implant containing an SRP would, on the one hand, induce adaptive immunity against the SRP as described in the '733 patent when the implant is provided one day after hatching or, on the other hand, induce immune tolerance against the SRP.

The Examiner suggests that the '479 patent answers this question by teaching that SRPs may be delivered *in ovo* at column 11, lines 12-17. However, the '479 does not teach doing so to eggs that, as recited in claim 34, contain "maternal antibody to the [SRP.]"

The combination of the '733 patent, the '479 patent, and the '766 patent fails to provide one skilled in the art with a reasonable expectation that injecting a biocompatible implant containing a selected immunogen *in ovo* would induce an effective adaptive immune response rather than inducing immune tolerance. Appellants therefore respectfully submit that claim 34 is patentable under 35 U.S.C. §103(a) over the combination of the '733 patent, the '479 patent, and the '766 patent and request that the rejection of claims 34, 37, 39-43, 67, 68, 97, and 100 be reversed.

2. Claims 69, 98, and 101 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,538,733), in view of Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766).

Claim 69 is independent. Each of claims 98 and 101 depends, directly or indirectly, from claim 69 and, therefore, includes all of the features recited in claim 69. Thus, remarks that refer to claim 69 apply equally to claims 98 and 101.

Claim 69 recites a method for inducing adaptive immunity in a bird against a selected immunogen. Generally, the method includes injecting a biocompatible implant *in ovo*, wherein the biocompatible implant comprises the selected immunogen and a biocompatible matrix material, and hatching eggs to result in birds, wherein the eggs comprise maternal antibody to the immunogen, wherein the implant provides for sustained release of the immunogen until a time when maternal antibodies of the birds to the immunogen are sufficiently reduced so that the birds are capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a bacterium.

Consequently, vaccinating a bird embryo *in ovo* according to the method recited in claim 69 presents the very same risks and, therefore, disincentives as doing so using the method recited in claim 34. Therefore, Appellants' remarks regarding the patentability of claims 34, 37, 39-43, 67, 68, 97, and 100 in the immediately preceding section are equally applicable to claims 69, 98, and 101.

Thus, the combination of the '733 patent, the '479 patent, and the '766 patent fails to provide one skilled in the art with a reasonable expectation that injecting a biocompatible implant containing a selected immunogen *in ovo* would induce an effective adaptive immune response rather than inducing immune tolerance. Appellants therefore respectfully submit that claim 69 is patentable under 35 U.S.C. §103(a) over the combination of the '733 patent, the '479 patent, and the '766 patent and request that the rejection of claims 69, 98, and 100 be reversed.

3. Claims 84-86, 89, 91-95, 99, and 102 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,538,733), in view of Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766).

Claim 84 is independent. Each of claims 85, 86, 89, 91-95, 99, and 102 depends, directly or indirectly, from claim 84 and, therefore, includes all of the features recited in claim

84. Thus, remarks that refer to claim 84 apply equally to each of claims 85, 86, 89, 91-95, 99, and 102.

Claim 84 recites a method for inducing adaptive immunity in a population of birds against a selected immunogen. Generally, the method includes injecting a biocompatible implant into a population of eggs that comprise maternal antibody to the selected immunogen, wherein the biocompatible implant comprises the selected immunogen and a biocompatible matrix material, wherein the implant provides for sustained release of the immunogen until the maternal antibodies to the immunogen in birds hatching from the eggs are reduced, and the birds hatched from the eggs are capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a bacterium.

Claim 84 differs from claims 34 and 69 in being directed to a method that includes injecting a biocompatible implant into a population of eggs. Thus, claim 84 emphasizes the value of the method to the poultry industry. Practicing the method of claim 84 allows one to vaccinate all of the eggs in a generation at one time and provide all of the birds that hatch from that generation of eggs with adaptive immunity to the selected immunogens contained in the implant, regardless of the qualitative and/or quantitative status of each egg with respect to maternal antibodies against the selected immunogen. In order to provide the same level of immunological protection to the generation of birds hatching from the eggs, one would have to either vaccinate the entire population repeatedly (to account for the varying amounts of maternally-derived antibody against the selected immunogen in different eggs in the generation) or vaccinate the entire population only after any maternally-derived antibody against the selected immunogen would have waned and, in doing so, leaving certain birds – i.e., those lacking maternally-derived antibodies against the selected immunogen – unprotected and immunologically vulnerable to infection by pathogens that express the selected immunogen.

Nevertheless, the very same risks and, therefore, disincentives for vaccinating using a sustained release implant *in ovo* exist in the context of vaccinating a population. Thus, Appellants' remarks regarding the patentability of claims 34, 37, 39-43, 67, 68, 97, and 100 and

claims 69, 98, and 101 in the immediately preceding two sections are equally applicable to claims 84-86, 89, 91-95, 99, and 102.

The combination of the '733 patent, the '479 patent, and the '766 patent fails to provide one skilled in the art with a reasonable expectation that injecting biocompatible implants containing a selected immunogen *in ovo* would induce effective adaptive immune responses rather than inducing immune tolerance in the birds hatching from the vaccinated eggs. Appellants therefore respectfully submit that claim 84 is patentable under 35 U.S.C. §103(a) over the combination of the '733 patent, the '479 patent, and the '766 patent and request that the rejection of claims 84-86, 89, 91-95, 99, and 102 be reversed.

B. Claims 34-44, 67-69, 71-82 and 84-102 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766) in view of Emery et al. (U.S. Patent No. 5,538,733), and further in view of Evans et al. (U.S. Patent No. 6,500,438)

1. Claims 34-44, 67, 68, 97, and 100 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766) in view of Emery et al. (U.S. Patent No. 5,538,733), and further in view of Evans et al. (U.S. Patent No. 6,500,438).

Claim 34 is independent. Each of claims 35-44, 67, 68, 97, and 100 depends, directly or indirectly, from claim 34 and, therefore, includes all of the features recited in claim 34. Thus, remarks that refer to claim 34 apply equally to claims 35-44, 67, 68, 97, and 100.

Claim 34 is summarized in part A.1 of this section of the Appeal Brief and, for brevity, will not be reiterated here. Similarly, the deficiencies of the combination of the '479 patent, '733 patent, and the '766 patent are provided in section A.1 of this section of the Appeal Brief and will not be reiterated here.

The '438 patent is cited for teaching *in ovo* vaccination of chickens in the final quarter of incubation. The '438 patent does not teach or suggest – and is not asserted to teach or suggest – vaccinating *in ovo* in the presence of maternal antibodies directed against immunogens contained in the vaccine. Accordingly, the '438 patent fails to cure the deficiencies of the combination of the '479 patent, '733 patent, and the '766 patent with respect to the subject matter of claim 34.

The combination of the '733 patent, the '479 patent, the '766 patent, and the '438 patent fails to provide one skilled in the art with a reasonable expectation that injecting a biocompatible implant containing a selected immunogen *in ovo* would induce an effective adaptive immune response rather than inducing immune tolerance. Appellants therefore respectfully submit that claim 34 is patentable under 35 U.S.C. §103(a) over the combination of the '733 patent, the '479 patent, the '766 patent, and the '438 patent and request that the rejection of claims 34-44, 67, 68, 97, and 100 be reversed.

2. Claims 69, 71-82, 98, and 101 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766) in view of Emery et al. (U.S. Patent No. 5,538,733), and further in view of Evans et al. (U.S. Patent No. 6,500,438).

Claim 69 is independent. Each of claims 71-82, 98, and 101 depends, directly or indirectly, from claim 69 and, therefore, includes all of the features recited in claim 69. Thus, remarks that refer to claim 69 apply equally to claims 71-82, 98, and 101.

Claim 69 is summarized in part A.2 of this section of the Appeal Brief and, for brevity, will not be reiterated here. Similarly, the deficiencies of the combination of the '479 patent, '733 patent, and the '766 patent are provided in section A.1 of this section of the Appeal Brief and will not be reiterated here.

The '438 patent is cited for teaching *in ovo* vaccination of chickens in the final quarter of incubation. The '438 patent does not teach or suggest – and is not asserted to teach or suggest – vaccinating *in ovo* in the presence of maternal antibodies directed against immunogens

contained in the vaccine. Accordingly, the '438 patent fails to cure the deficiencies of the combination of the '479 patent, '733 patent, and the '766 patent with respect to the subject matter of claim 69.

The combination of the '733 patent, the '479 patent, the '766 patent, and the '438 patent fails to provide one skilled in the art with a reasonable expectation that injecting a biocompatible implant containing a selected immunogen *in ovo* would induce an effective adaptive immune response rather than inducing immune tolerance. Appellants therefore respectfully submit that claim 69 is patentable under 35 U.S.C. §103(a) over the combination of the '733 patent, the '479 patent, the '766 patent, and the '438 patent and request that the rejection of claims 69, 71-82, 98, and 101 be reversed.

3. Claims 84-96, 99, and 102 are patentable under 35 U.S.C. §103(a) over Emery et al. (U.S. Patent No. 5,830,479) in view of Phelps et al. (U.S. Patent No. 5,339,766) in view of Emery et al. (U.S. Patent No. 5,538,733), and further in view of Evans et al. (U.S. Patent No. 6,500,438).

Claim 84 is independent. Each of claims 85-96, 99, and 102 depends, directly or indirectly, from claim 84 and, therefore, includes all of the features recited in claim 84. Thus, remarks that refer to claim 84 apply equally to each of claims 85-96, 99, and 102.

Claim 84 is summarized in part A.3 of this section of the Appeal Brief and, for brevity, will not be reiterated here. Similarly, the deficiencies of the combination of the '479 patent, '733 patent, and the '766 patent are provided in section A.1 of this section of the Appeal Brief and will not be reiterated here.

The '438 patent is cited for teaching *in ovo* vaccination of chickens in the final quarter of incubation. The '438 patent does not teach or suggest – and is not asserted to teach or suggest – vaccinating *in ovo* in the presence of maternal antibodies directed against immunogens contained in the vaccine. Accordingly, the '438 patent fails to cure the deficiencies of the combination of the '479 patent, '733 patent, and the '766 patent with respect to the subject matter of claim 84.

The combination of the '733 patent, the '479 patent, the '766 patent, and the '438 patent fails to provide one skilled in the art with a reasonable expectation that injecting a biocompatible implant containing a selected immunogen *in ovo* would induce an effective adaptive immune response rather than inducing immune tolerance. Appellants therefore respectfully submit that claim 84 is patentable under 35 U.S.C. §103(a) over the combination of the '733 patent, the '479 patent, the '766 patent, and the '438 patent and request that the rejection of claims 84-96, 99, and 102 be reversed.

VIII. SUMMARY

For the foregoing reasons, Appellant respectfully requests that the Board review and reverse the rejection of claims 34-44, 67-69, 71-82, and 84-102 as discussed herein and that notification of the allowance of these claims be issued.

Respectfully submitted
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CERTIFICATE UNDER 37 CFR §1.8:

The undersigned hereby certifies that this paper is being transmitted via the U.S. Patent and Trademark Office electronic filing system in accordance with 37 CFR §1.6(a)(4) to the Patent and Trademark Office addressed to Mail Stop - Appeal Brief - Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on this 15th day of December, 2009.

By: Deb Schurmann
Name: Deb Schurmann

CLAIMS APPENDIX

Serial No. 10/749,602

Docket No. 293.00010102

Claims 34-44, 67-69, 71-82 and 84-102 are provided below.

1-33. (Canceled)

34. (Rejected) A method for inducing adaptive immunity in a bird against a selected immunogen comprising:

injecting a biocompatible implant into an egg, wherein the biocompatible implant comprises the selected immunogen and a biocompatible matrix material, wherein the egg comprises maternal antibody to the selected immunogen, wherein the implant provides for sustained release of the immunogen until the maternal antibodies in a bird hatching from the egg are reduced so that the bird is capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a gram-negative bacterium.

35. (Rejected) The method according to claim 34, wherein the implant is injected during the fourth quarter of incubation of an egg.

36. (Rejected) The method according to claim 34, wherein the implant is injected at about 15-28 days of incubation of an egg.

37. (Rejected) The method according to claim 34, wherein the bird is selected from the group consisting of turkey, chicken, duck, goose, ostrich and pheasant.

38. (Rejected) The method according to claim 34, wherein the bird is a turkey and the implant is injected at about 25-27 days of incubation of an egg.

39. (Rejected) The method according to claim 34, wherein the implant provides for sustained release of the immunogen for about 1-90 days post-hatching.

40. (Rejected) The method according to claim 34, wherein the implant provides for sustained release of the immunogen for about 1-60 days post-hatching.

41. (Rejected) The method according to claim 34, wherein the implant provides for sustained release of the immunogen for about 1-35 days post-hatching.

42. (Rejected) The method according to claim 34, wherein the implant is injected at about 25-27 days of incubation of an egg and wherein the implant provides for sustained release of the immunogen for about 1-90 days post-hatching of the egg.

43. (Rejected) The method according to claim 34, further comprising administering a second dose of the immunogen at 3-12 weeks post hatching to stimulate a secondary immune response.

44. (Rejected) The method according to claim 34, wherein the bird is a chicken and the implant is injected at about day 17 to 19 of incubation of an egg.

45-66. (Canceled)

67. (Rejected) The method according to claim 34, wherein the implant further provides for delayed release.

68. (Rejected) The method according to claim 34, wherein the immunogen further comprises a porin protein.

69. (Rejected) A method for inducing adaptive immunity in a bird against a selected immunogen comprising:

injecting a biocompatible implant *in ovo*, wherein the biocompatible implant comprises the selected immunogen and a biocompatible matrix material, and hatching eggs to result in birds, wherein the eggs comprise maternal antibody to the immunogen, wherein the implant provides for sustained release of the immunogen until a time when maternal antibodies of the birds to the immunogen are sufficiently reduced so that the birds are capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a bacterium.

70. (Canceled)

71. (Rejected) The method according to claim 69, wherein the implant further provides for delayed release.

72. (Rejected) The method according to claim 69, wherein the immunogen further comprises a porin protein.

73. (Rejected) The method according to claim 69, wherein the implant is injected during the fourth quarter of incubation of an egg.

74. (Rejected) The method according to claim 69, wherein the implant is injected at about 15-28 days of incubation of an egg.

75. (Rejected) The method according to claim 69, wherein the bird is selected from the group consisting of turkey, chicken, duck, goose, ostrich and pheasant.

76. (Rejected) The method according to claim 69, wherein the bird is a turkey and the implant is injected at about 25-27 days of incubation of an egg.

77. (Rejected) The method according to claim 69, wherein the implant provides for sustained release of the immunogen for about 1-90 days post-hatching.

78. (Rejected) The method according to claim 69, wherein the implant provides for sustained release of the immunogen for about 1-60 days post-hatching.

79. (Rejected) The method according to claim 69, wherein the implant provides for sustained release of the immunogen for about 1-35 days post-hatching.

80. (Rejected) The method according to claim 69, wherein the implant is injected at about 25-27 days of incubation of an egg and wherein the implant provides for sustained release of the immunogen for about 1-90 days post-hatching of the egg.

81. (Rejected) The method according to claim 69, further comprising administering a second dose of the immunogen at 3-12 weeks post hatching to stimulate a secondary immune response.

82. (Rejected) The method according to claim 69, wherein the bird is a chicken and the implant is injected at about day 17 to 19 of incubation of an egg.

83. (Canceled)

84. (Rejected) A method for inducing adaptive immunity in a population of birds against a selected immunogen comprising:

injecting a biocompatible implant into a population of eggs that comprise maternal antibody to the selected immunogen, wherein the biocompatible implant comprises the selected

immunogen and a biocompatible matrix material, wherein the implant provides for sustained release of the immunogen until the maternal antibodies to the immunogen in birds hatching from the eggs are reduced, and the birds hatched from the eggs are capable of mounting an adaptive immune response to the immunogen, wherein the immunogen comprises a siderophore receptor protein from a bacterium.

85. (Rejected) The method according to claim 84, wherein the implant further provides for delayed release.

86. (Rejected) The method according to claim 84, wherein the immunogen further comprises a porin protein.

87. (Rejected) The method according to claim 84, wherein the implant is injected during the fourth quarter of incubation of an egg.

88. (Rejected) The method according to claim 84, wherein the implant is injected at about 15-28 days of incubation of an egg.

89. (Rejected) The method according to claim 84, wherein the bird is selected from the group consisting of turkey, chicken, duck, goose, ostrich and pheasant.

90. (Rejected) The method according to claim 84, wherein the bird is a turkey and the implant is injected at about 25-27 days of incubation of an egg.

91. (Rejected) The method according to claim 84, wherein the implant provides for sustained release of the immunogen for about 1-90 days post-hatching.

92. (Rejected) The method according to claim 84, wherein the implant provides for sustained release of the immunogen for about 1-60 days post-hatching.

93. (Rejected) The method according to claim 84, wherein the implant provides for sustained release of the immunogen for about 1-35 days post-hatching.

94. (Rejected) The method according to claim 84, wherein the implant is injected at about 25-27 days of incubation of an egg and wherein the implant provides for sustained release of the immunogen for about 1-90 days post-hatching of the egg.

95. (Rejected) The method according to claim 84, further comprising administering a second dose of the immunogen at 3-12 weeks post hatching to stimulate a secondary immune response.

96. (Rejected) The method according to claim 84, wherein the bird is a chicken and the implant is injected at about day 17 to 19 of incubation of an egg.

97. (Rejected) The method according to claim 34 wherein the implant further comprises an adjuvant.

98. (Rejected) The method according to claim 69 wherein the implant further comprises an adjuvant.

99. (Rejected) The method according to claim 84 wherein the implant further comprises an adjuvant.

100. (Rejected) The method according to claim 43 wherein the administering a second dose of the immunogen comprises administering a modified live vaccine.

101. (Rejected) The method according to claim 81 wherein the administering a second dose of the immunogen comprises administering a modified live vaccine.

102. (Rejected) The method according to claim 95 wherein the administering a second dose of the immunogen comprises administering a modified live vaccine.

EVIDENCE APPENDIX

Serial No. 10/749,602

Docket No. 293.00010102

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1. U.S. Patent No. 5,339,766, entered into the record by citation within the Information Disclosure Statement filed March 9, 2003.
2. U.S. Patent No. 5,538,733, entered into the record by citation within the Information Disclosure Statement filed March 9, 2003.
3. U.S. Patent No. 5,830,479, entered into the record by citation within the Information Disclosure Statement filed March 9, 2003.
4. U.S. Patent No. 6,500,438, entered into the record by citation within the Notice of References Cited issued July 28, 2005.
5. Final Office Action, issued August 20, 2009.
6. Response, filed May 7, 2009, to Non-Final Office Action issued January 7, 2009.
7. Davis *et al.* eds., *Microbiology*, fourth edition, 1990, J.B. Lippincott Co., Philadelphia, Pennsylvania, pp. 381-382, entered into the record by citation within the Information Disclosure Statement filed May 7, 2009.

US005339766A

United States Patent [19]

Phelps et al.

[11] Patent Number: 5,339,766

[45] Date of Patent: Aug. 23, 1994

[54] METHOD OF INTRODUCING MATERIAL INTO EGGS DURING EARLY EMBRYONIC DEVELOPMENT

[75] Inventors: Patricia V. Phelps, Raleigh; Julius K. Tyczkowski, Cary; Toni O. O'Connell, Zebulon; Ann B. Gore, Raleigh, all of N.C.

[73] Assignee: Embrex, Inc., Research Triangle Park, N.C.

[21] Appl. No.: 147,162

[22] Filed: Nov. 3, 1993

[51] Int. Cl.[5] A01K 67/00

[52] U.S. Cl. 119/6.8

[58] Field of Search 119/6.8, 6.6, 174

[56] References Cited

U.S. PATENT DOCUMENTS

2,477,752	8/1949	Kiss .	
2,734,482	2/1956	Seltzer .	
3,256,856	6/1966	Nicely et al. .	
3,377,989	4/1968	Sandhage et al. .	
4,040,388	8/1977	Miller .	
4,469,047	9/1984	Miller	119/6.8
4,593,646	6/1986	Miller et al. .	
4,681,063	7/1987	Hebrank .	
4,903,635	2/1990	Hebrank .	
4,928,628	5/1990	Gassman et al. .	
4,928,629	5/1990	Trampel .	
5,056,464	10/1991	Lewis .	
5,136,979	8/1992	Paul et al. .	
5,176,101	6/1993	Paul et al. .	
5,206,015	5/1993	Cox et al.	119/6.8

Primary Examiner—John G. Weiss

Attorney, Agent, or Firm—Bell, Seltzer, Park & Gibson

[57] ABSTRACT

A method of introducing a substance into a bird egg through the shell thereof comprises the steps of applying a seal to the exterior of the shell, inserting an injection device through the seal and into the egg, injecting the substance through the injection device and into the interior of the egg, and withdrawing the injection device from the egg through the seal. The method is particularly preferred for injecting substances into the albumin during the first quarter of injection through the bottom of the egg when the small end of the egg is oriented downward to prevent the introduction of air bubbles through the opening formed in the shell by the injection device.

22 Claims, 1 Drawing Sheet

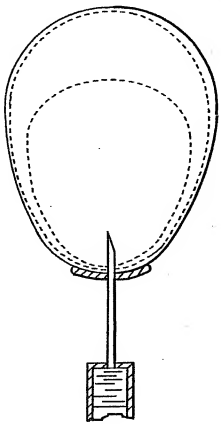
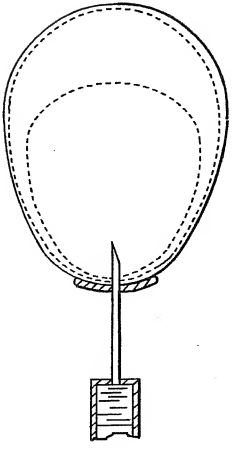


FIG. 1

FIG. 3



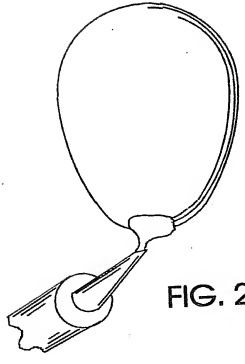


FIG. 2

METHOD OF INTRODUCING MATERIAL INTO EGGS DURING EARLY EMBRYONIC DEVELOPMENT

FIELD OF THE INVENTION

The present invention relates to a method of introducing material into eggs during early embryonic development.

BACKGROUND OF THE INVENTION

The desirability of injecting materials into avian eggs during incubation has been recognized for some time. Initially, the purpose of injecting eggs was to prepare various vaccines using the egg as a growth medium for the vaccine. More recent developments have involved injecting live embryonated eggs for the purpose of accomplishing some beneficial or therapeutic effect on the embryo or the bird that eventually hatches from the egg. Such beneficial effects include increased growth, disease resistance due to in ovo vaccination, increased percentage hatch of multiple incubated eggs, and otherwise improved physical characteristics of hatched poultry.

Several basic techniques and injection devices for injecting materials into live embryonated eggs have been described, including forcing fluids through the egg shell using pressurization and physically forming an opening in the shell of an egg and then adding the desired material (e.g., injection using syringe and needle arrangements). One traditional method has been syringe injection of eggs by hand.

Several injection devices seal the injection hole after injection to prevent leakage and contamination. U.S. Pat. No. 4,593,646 to Miller et al. discloses a method and apparatus for automatic egg injection in which support plates hold and properly position a plurality of injection devices and eggs. Each egg is sealed after injection by heat coagulating the albumin located near the injection hole. An additional sealant is then applied to the outer shell by dipping each egg into a bath of the sealant. The '646 patent does not disclose sealing the egg prior to injection.

U.S. Pat. No. 4,040,388 to Miller discloses a method and apparatus for automatic egg injection in which the downwardly facing small end of an egg is punctured. The portion of the device which punctures the egg is heated in the '388 method, allegedly sterilizing the exterior of the egg (thus preventing infection during injection) and also sealing the hole by heat coagulating a small portion of the egg albumin. The '388 patent does not disclose sealing the egg prior to injection.

U.S. Pat. No. 2,477,752 to Kiss discloses a method of injecting fertile eggs for the purpose of producing chicks having down of predetermined colors. The '752 patent discloses injecting the egg manually with a syringe and thereafter by sealing the opening in the egg. While the patent states that care should be taken to prevent air from entering the egg, no method for preventing the entrance of air is provided. Sealing the egg prior to injection is not disclosed.

U.S. Pat. No. 5,136,979 to Paul et al. discloses an apparatus and method for injecting a plurality of eggs to the same depth and location even when the eggs are of varying sizes and are misaligned. The apparatus includes a means for sterilizing the egg punch and needle sections after each injection. U.S. Pat. No. 5,056,464 to Lewis discloses an apparatus and method for injecting a plurality of eggs in which a suction cup apparatus is used for grasping each egg. U.S. Pat. No. 4,903,635 to Hebrank discloses a high speed automated injection system in which eggs are lifted using suction devices and separate devices are used for forming an opening in the egg shell and for injecting a fluid substance.

Some egg injection devices deliver material through the small end of an egg into the albumin. Injecting material through the large end of an egg and into the air sac above the albumin is not appropriate for delivery of all materials. Delivery into the albumin, however, increases the risk of leakage of albumin and ingress of air and contaminants after injection. Methods of injecting material into the albumin of eggs on a rapid basis should preferably provide means for preventing air and contaminants from entering the albumin, and means for preventing leakage of albumin, after injection.

SUMMARY OF THE INVENTION

A first aspect of the present invention is a method of injecting a desired substance into an avian egg through the egg shell, comprising applying sealant to the exterior of the shell at the injection site, inserting an injection device through the sealant and into the egg (preferably the albumin of the egg), injecting a preselected substance into the egg (preferably the albumin of the egg), and withdrawing the injection device from the egg. The sealant serves to seal the opening which would otherwise remain upon withdrawal of the injection device. The egg may then be incubated to hatch.

In one embodiment of the present invention, the applying step comprises the steps of, first, depositing a liquid sealant material on the egg, and then curing the liquid sealant on the egg to form an elastic seal thereon. In another embodiment of the invention, the applying step comprises the steps of, first, providing a preformed elastic seal member, and then adhering the preformed elastic seal member to the egg.

The foregoing and other objects and aspects of the present invention are explained in detail in the drawings herein and the specification set forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic view of an arian egg showing the proper orientation of an egg during incubation, with the large end of the egg pointing upwards, as such an egg may be oriented for carrying out the present invention.

FIG. 2 shows the deposition of sealant on an egg prior to insertion of an injection device in accordance with the present invention.

FIG. 3 shows an egg with an injection device inserted through the sealant and into the albumin of the egg in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes a method for injecting eggs to minimize the ingress of air and contaminants, and minimize the leakage of albumin from the egg.

To inject eggs using the method of the present invention, a sealant is first applied at the site of injection. While injection may occur at any site whereby the material injected is placed in the albumin, a particularly preferred site is the small end (bottom) of the egg. Sealant may be any suitable resilient or elastic material which allows the needle to be inserted through the

sealant while essentially preventing the entry of air through the injection site into the egg during injection and subsequent incubation. Examples of suitable sealants include silicone sealants (e.g., G.E. TM Silicone II), adhesives or glues (e.g., DUCO TM cement, mucilage glue), hot melt adhesives, or any other liquid which solidifies and hardens after application and through which a needle can be inserted, yet which retains sufficient elasticity that a syringe or needle can be inserted and withdrawn therethrough with subsequent sealing of the hole made thereby. Alternatively, a glue or adhesive may be used to attach another component to the egg through which the injection occurs, and which acts to prevent air entry into the egg (e.g., a rubber or silicone septum attached firmly to the egg surface).

FIG. 1 is a schematic depiction of an egg with the small end (bottom) 8 oriented downward and the large end (top) 9 oriented upward. As shown in FIG. 2, in practicing the present invention, sealant (10) is applied to the egg shell at the site of injection. As shown in FIG. 3, after the sealant (10) is applied to the egg surface, an injection needle (11) or other injection device is inserted through the sealant and underlying egg shell (12) and membranes (13), and the material to be injected (14) is expelled in the interior of the egg. In general, a portion of the shell is left unsealed, which unsealed portion is sufficient in size to permit respiration of the embryo within the egg during incubation so that the embryo may be incubated to hatch. Preferably a major portion of the egg shell remains unsealed, with the sealed portion preferably including the small end (bottom) of the egg.

The materials or substances to be delivered include, but are not limited to vaccines, vitamins, antibiotics, hormones, enzyme inhibitors, peptides, cells, DNA, and other therapeutic molecules. Materials or substances may be fluids, liquids, solutions, liquid-liquid suspensions, liquid-solid suspensions, gases, gaseous suspensions, emulsions, and solid materials such as biodegradable polymers (e.g., in the form of syringeable beads) which release active agents such as described above upon biodegredation. Examples of biodegradable polymers include, but are not limited to, polylactide polymers, including lactic/glycolide copolymers. See, e.g., U.S. Pat. Nos. 3,773,919, 4,568,559, and 4,389,330 (the disclosures of which applicant specifically intend to be incorporated herein by reference).

The surface of the egg may optionally be sanitized prior to injection by any suitable method. Examples of suitable sanitizing compounds include formaldehyde, H_2O_2 (e.g., 3% H_2O_2, 5% H_2O_2, 5% H_2O_2 combined with a quaternary ammonium agent), chlorine based sanitizers, or other commercially available egg sanitizers. Any suitable method of applying a sanitizing compound may be used, including but not limited to fumigation, microaerosol fumigation, dipping of whole egg, direct application to egg, vacuum application to egg, or other techniques as are known in the art.

The seal may carry or contain an antibacterial agent such as GARISOL TM, so that when the needle or injection device is inserted through the seal it is sanitized by contact to the antibacterial agent contained in or carried by the seal.

The term "birds" as used herein, is intended to include males or females of any avian species, but is primarily intended to encompass poultry which are commercially raised for eggs or meat. Accordingly, the term "bird" is particularly intended to encompass hens, cocks and drakes of chickens, turkeys, ducks, geese, quail, ostriches and pheasants. Chickens and turkeys are preferred.

The term "in ovo," as used herein, refers to birds contained within an egg prior to hatch. Thus, the present invention may be conceived of as both a method of introducing a compound into an egg as well as a method of administering a substance to a bird. The present invention may be practiced with any type of bird egg, including chicken, turkey, duck, goose, quail, ostrich and pheasant eggs. Chicken and turkey eggs are preferred. Any region within the egg may be injected, including the region defined by the amnion (including the embryo), the albumin, and the yolk sac (preferably the albumin). Eggs treated by the method of the present invention are preferably fertile eggs which may be in any period of incnbation, from early to late, but are preferably in the first half of incubation. More preferably, eggs are in the first quarter of incubation. Selection of the time of injection will depend upon the agent being injected and the desired effect, and will be ascertainable by one skilled in the art.

The term "Day 0" as used herein refers to eggs prior to incubation.

The present invention is particularly advantageous for injecting the albumin of eggs during early embryonic development without significantly decreasing hatchability. When injecting early embryonic eggs in the albumin, introduction of an air bubble can interfere with the development of the embryo and result in decreased hatchability rates. The method of the present invention allows injection into the albumin at Day 0 of incubation with good hatchability rates. The use of a sealant prior to injection essentially prevents the introduction of air into the egg and reduces bacterial contamination caused by the introduction of a needle and exposure of internal egg contents to the external environment.

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In these examples, μl means microliter, ml means milliliter, mg means milligram, and (CFU means colony forming units).

EXAMPLE 1

Decreased Hatchability Due to Air Bubble

In initial attempts to inject Day 0 eggs, the eggs were injected through the small end while held upside down (small end up); this resulted in hatchability rates of from 5–8%, regardless of the sanitation measures used on the eggs prior to injection (data not shown). Using illumination and visual examination of injected eggs, it was noted that eggs injected in this manner were developing abnormally, for example, eggs were noted to have frothy albumin, absent aircells, or aircells that moved as the egg was rotated. Illumination and examination of eggs during injection revealed that an air bubble entered the egg through the injection site. If the egg was inverted during injection and then turned right-side up for incubation, the bubble rose and rested between the inner shell membrane and the developing blastodisc (early embryo). When the egg was injected through the small end while held upright, either no air bubble entered the egg or it did not rise to the top of the egg. Hatchability data of noninjected control eggs, eggs injected (small end) while inverted, and eggs injected (small end) while held upright are shown in Table 1.

TABLE 1

TREATMENT	Hatchability Following Injection[1]. AIR BUBBLE INTERFERENCE	HATCHABILITY	EGGS OBSERVED (n)
NONINJECTED CONTROLS	NO	83%	100
BOTTOM (small end) INJECTED; EGGS INVERTED	YES	4%	100
BOTTOM (small end) INJECTED; EGGS UPRIGHT*	NO	79%	94

[1]Eggs were turned upside down and then righted before injection to mimic mechanical handling of eggs injected upside down. All eggs were sealed after injection with silicone sealant (G.E. TM Silicon II).

A similar injection protocol was used on Day 5 of incubation (data not shown), and resulted in increased hatchability over injection at Day 0.

These results indicate that injection into the albumin via the small end of the egg during early embryonic development can cause an air bubble which interferes with embryonic development. While not wishing to be held to any single theory, the inventors hypothesize that an air bubble resting between the blastodisc and inner shell membrane (floor of the aircell) interferes with formation of the chorioallantoic vasculature system and early respiration of the embryo. By Day 5 of incubation, the chorioallantoic vascular system has already begun to fuse to the innershell membrane, and the effects of the air bubbles would not be as severe.

EXAMPLE 2

Sealing Eggs Prior to Injection Using a Septum

The use of a rubber septum attached to the eggshell, through which the injection would occur, was investigated to determine if this would prevent the entrance of an air bubble during injection. Approximately 100 eggs were examined. A small rubber gas chromatography septum (Fisher Scientific) approximately 5 millimeters in diameter, was attached to the small end of each egg with adhesive. Each egg was injected through the septum at Day 0, while the egg was held upright. Eggs were illuminated and observed on Day 11, and no abnormal development (e.g., frothy albumin, absent air cell, or moving air cell) was observed.

These results indicate that injection of early embryonic eggs through a rubber septum prevented the entrance of an air bubble during injection.

EXAMPLE 3

Comparison of Sealing Prior to and After Injection

A pilot study to compare the use of sealant prior to and after injection was carried out. It was hypothesized that hatchability of eggs sealed and then injected might be improved over eggs injected and then sealed. It was hypothesized that sealant, when applied prior to injection, would cleanse the needle during entry into the egg and forestall entry of outside contaminants as well as prevent the entry of air during injection.

Three treatment groups of 60 eggs each were used: non-injected control eggs; eggs injected (small end) while upright and then sealed (injected-sealed group); eggs sealed and then injected (small end) while upright (sealed-injected group). All eggs were sanitized by dipping into a solution of 5% H_2O_2 prior to injection. All eggs were injected on day 0 of incubation with a 20 gauge needle inserted into the small end of the egg while the egg was held in an upright (large end up) position, and eggs were then incubated to hatch in a routine manner. No vehicle was injected into the eggs.

Silicone sealant (G.E. TM Silicone II) was used as the sealant. For sealed-injected eggs, a small amount of sealant (approximately the size of a small pea) was placed on the egg shell at the site of injection, allowed to dry, and the needle inserted through the sealant. The amount of sealant used was such that the needle was inserted through a sealant layer between about 1 millimeter and 3 millimeters thick, and which surrounded the needle. For injected-sealed eggs, the injection was performed and, after the needle was withdrawn, sealant was placed over the injection site.

As seen in Table 2, hatchability of injected-sealed eggs was 86% of non-injected controls, while the hatchability of sealed-injected eggs was actually greater than (105% of) the non-injected controls. These results suggest that injecting eggs upright through a sealant improves hatchability over eggs injected upright and then sealed.

TABLE 2

Day 0 Injection: Hatchability of Eggs Inlected Upright Treatment	Hatchability (Percent of Noninjected Controls)
Noninjected n = 60	100
Injected-sealed n = 60	86
Sealed-injected n = 60	105

EXAMPLE 4

Effect of Needle Size and Volume Injected

To determine whether needle size adversely affected hatchability after Day 0 injection, punches of 16 gauge, 18 gauge, 20 gauge and 22 gauge were used. No material was injected into the eggs. The eggs utilized in this experiment were from an older breeder flock exhibiting highly variable percentages of infertiles and early embryonic mortality. However, the results shown in Table 3 indicate a trend for decreased hatchability with increased needle or punch size.

TABLE 3

Day 0 Injection: Effects of Needle Size on Hatchability[1] Treatment	Hatchability %	Early Deads	Middle Deads	Late Deads	Live Pips
Noninjected	75	11	0	3	4
Noninjected + Sealant	81	5	0	3	3
22 Gange Punch	72	11	1	2	4
20 Gauge Punch	67	12	2	3	4
18 Gauge Punch	60	15	2	3	6
16 Gauge Punch	66	11	2	4	6

[1]Hatchability is based on chicks which hatched from all eggs set and includes infertiles, malpositions and malformations.

EXAMPLE 5

Decontamination of Needle by Sealant

Experiments were performed to test whether insertion of the injection needle through the sealant contributed to needle sanitation, thereby reducing egg-to-egg bacterial contamination when a needle was used for multiple injections. A needle was dipped in a broth

culture of *Escherichia coli* and then either inserted into a vial containing 1 ml of sterile water or into a vial containing 1 ml of sterile water which had been covered by a plug of silicone gel (G.E. TM Silicone II) similar in amount to that used on eggs prior to injection (see Example 3). To determine if antibiotic in the sealant would improve needle sanitation, in an additional treatment the needle was inserted through silicone sealant (G.E. TM Silicone II) into which GARASOL TM (Schering Corp., Kenilworth, N.J.) had been mixed at a concentration of 1 mg/gram (0.0625 ml GARASOL TM /6.25 grams of silicone). Results are presented in Table 4.

TABLE 4

Injection through Silicone and Decontamination of *E. Coli* Contaminated Needles

Untreated Needle Contamination Mean[1] CFU/needle	Needle Inserted Through Silicone Mean CFU/needle	Needle Inserted Through Silicone Containing GARASOL TM Mean CFU/needle
90,000,000	<1000	4,900,00
86,000,000	<1000	12,800,00
89,000,000	8,100,000	4,300,000

[1]Each mean represents serial dilutions of one needle tested. The minimum detection limit for this experiment was 1000 Colony Forming Unit (CFU).

The data in Table 4 suggest that inserting a needle through silicone sealant during egg injection significantly reduces contamination (~3 logs) of the needle even in the face of a high bacterial challenge. Interestingly, although the silicone sealant mixed with GARASOL TM (Schering Corp., Kenilworth, N.J.) reduced the bacterial load on the needle, it only reduced it by one log. Possibly the addition of antibiotic altered the consistency of the silicone, rendering it less effective in its sanitizing properties.

EXAMPLE 6

Delivery of Compounds at Day 0

As not all compounds designed for injection will be soluble in water or PBS, experiments were designed to indicate possible problems regarding early egg injection techniques using various excipients. These experiments were not intended to clearly discern small differences in hatchability. Treatment groups consisted of 120 eggs per group (two trials of 60 eggs per trial). Isopropanol was chosen as a diluent because many lipophilic or hydrophobic compounds which are insoluble in water can initially be solubilized in isopropanol and then diluted in water. Silica was incorporated into the experimental design as a representative polymer (inclusion of polymers in excipients allows proteins to adhere to the polymer resulting in a slow release formulation as well as increasing the antigenicity of antigens). Also tested as an excipient was MOLECUSOL TM (Pharmatec, Inc.) which is a beta-cyclodextran.

Hatchability was evaluated following injection of 50 µl of each excipient tested (Table 5). Day 0 broiler eggs were injected in the small end while held upright; sealant was applied to the injection site prior to injection (see Example 3). None of the excipients tested significantly depressed hatchability.

TABLE 5

Day 0 Injection:
Hatchability Following Administration of Vehicles

Treatment Group	hatchability (means[1])	early deads	middle deads	late deads
Noninjected control	79.7	7.4	1.65	0.8
Silica in 5% Isopropanol	78.7	8.1	1.1	0
Silica in 20% isopropanol	82.1	5.9	1	1.9
Molecusol 1%	84.8	9.9	1.5	0
Molecusol 5%	82.5	9.2	1	3.0
isopropanol 5%	81.8	7.3	0	3.2
isopropanol 20%	75.1	9.3	1.5	0

[1]Means are based on two trials each consisting of 60 eggs per treatment group (n = 120).

The results set forth in Table 5 indicate that multiple vehicles are suitable for use in the injection technique described above.

A further experiment using the quinolone antibiotic Sarafloxacin (Abbott Laboratories) was performed. Sarafloxacin is administered subcutaneously to day old chicks at 0.1 mg for control of bacterially related early mortality. A stock sarafloxacin solution, 50 mg/ml, (provided by Abbott Laboratories) was diluted in sterile water to concentrations of 5.0, 1.0, 0.5 and 0.1 mg/ml. Injection of 50 µl delivered 0.25, 0.05, 0.025 and 0.005 mg/egg, respectively. The doses injected at Day 0 were 2.5×, 0.5×, 0.25× and 0.05× the dose given to chicks at hatch. Injection was performed on Day 0 of incubation through silicone sealant (G.E. TM Silicone II) applied to the small end of eggs; eggs were held upright during injection (see Example 3).

TABLE 6

Day 0 Injection:
Hatchability Following Sarafloxacin Administration

Treatment Group	Hatchability (means %)[1]	Early Deads	Middle Deads	Late Deads
Noninjected Control	88.0	3.4	.80	1.6
Vehicle	89.7	6.75	.85	0
0.25 mg/egg (2.5 ×)	84.7	5.8	.80	1.6
0.05 mg/egg (0.5 ×)	86.6	4.9	.80	0
0.025 mg/egg (0.25 ×)	78.5	5.0	1.65	3.35
0.05 mg/egg (0.05 ×)	82.7	5.95	.85	1.7

[1]Percents based on normal hatched chicks divided by total eggs set; Means are based on two hatchability trials each consisting of 60 eggs per treatment group (n = 120).

Although all doses of Sarafloxacin administered resulted in a slight depression in hatchability, the effects were inconsistent across trials and the means do not demonstrate dose dependent effects since some lower doses depressed hatchability more than the maximum dose administered. These results indicate that the injection technique described above is capable of delivering an active agent without significantly depressing hatchability.

EXAMPLE 7

Combined Sanitation and Injection at Day 0

A technique combining sanitation of the egg shell injection site with injection through a sealant was tested to determine effects on hatchability as compared to non-injected controls. The small end of each egg to be injected was dipped in either a 5% H_2O_2/quaternary

ammonium sanitizer, or a chlorine based sanitizer consisting of 0.5% CHLOROX ™, for 4 minntes. A small dab (approximately the size of a small pea) of silicone sealant (G.E. ™ Silicone II) was then applied to the injection site on the small end, and then 50 μl of saline was injected through the sealant into the egg albumin using a 22 gauge needle, with the egg held upright. The data from two trials is presented in Table 7. Each treatment group used 300 eggs (two trials of 150 eggs).

TABLE 7

Group	Injected	Sanitized	Hatch %	Bacteria Contaminated Eggs %	Mold Contaminated Eggs %	Bacteria Contaminated Yolk Sacs %
1	No	No	79	4.7	1.3	4.6
2	No	Yes	76	1.4	0.7	0.5
3	Yes	No	78	4.0	1.3	8.5
4	Yes	Yes	77	5.3	0	2.0

The data set forth in Table 7 show that injected eggs have hatchability rates equivalent to noninjected controls. While sanitation of injected eggs does not appear to decrease the rate of bacterially contaminated eggs over non-sanitized injected eggs, the rate of mold contamination and bacterially contaminated yolk sacs of hatched chicks appeared to be reduced in sanitized injected eggs over non-sanitized injected eggs.

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That which is claimed is:

1. A method of introducing a substance into a bird egg through the shell thereof, comprising the steps of:

applying a seal to the exterior of the shell;

inserting an injection device through said seal and into the egg;

injecting said substance through said injection device and into the interior of the egg; and

withdrawing said injection device from the egg through said seal;

whereby said substance is deposited in the interior of the egg.

2. A method as in claim **1** wherein said seal is applied to the small end of the egg.

3. A method as in claim **1** wherein said egg is oriented with the small end down and said injection device travels upwardly through the egg shell at the small end thereof and into the interior of the egg.

4. A method as in claim **1** wherein said substance injected is a liquid.

5. A method as in claim **1** wherein said substance injected is a solid.

6. A method as in claim **1** wherein said withdrawing step is followed by the step of incubating said egg to hatch.

7. A method as in claim **1** wherein the egg is selected from the group consisting of turkey eggs, chicken eggs, quail eggs, duck eggs, goose eggs, ostrich eggs and pheasant eggs.

8. A method as in claim **1** wherein said egg is injected during the first quarter of incubation.

9. A method as in claim **1** wherein said egg is injected prior to the first day of incubation.

10. A method as in claim **1**, wherein said applying step comprises the steps of:

depositing a liquid sealant material on the egg; and then

curing said liquid sealant on the egg to form an elastic seal thereon.

11. A method as in claim **1**, wherein said applying step comprises the steps of:

providing a preformed elastic seal member; and then

adhering said preformed elastic seal member to said egg.

12. A method as in claim **1**, wherein said substance is deposited in the interior of the egg in the region defined by the amnion, the albumin or the yolk sac.

13. A method of introducing a substance into a bird egg through the shell thereof, comprising:

applying a seal to the exterior of the shell;

inserting an injection device through said seal and into the egg, which egg is in the first quarter of incubation thereof;

injecting said substance through said injection device and into the region of the egg defined by the albumin;

withdrawing said injection device from the egg through said seal; and then

incubating said egg to hatch.

14. A method as in claim **13** wherein said seal is applied to the small end of said egg.

15. A method as in claim **13** wherein said egg is oriented with the small end down and said injection device travels upwardly through the egg shell at the small end thereof and into the interior of the egg.

16. A method as in claim **13** wherein said material injected into said egg is a liquid.

17. A method as in claim **13** wherein said material injected into said egg is a solid.

18. A method as in claim **13** wherein said egg is selected from the group consisting of turkey eggs, chicken eggs, quail eggs, duck eggs, goose eggs, ostrich eggs and pheasant eggs.

19. A method as in claim **13** wherein said egg is injected prior to the first day of incubation.

20. A method as in claim **13**, wherein said applying step comprises the steps of:

depositing a liquid sealant material on the egg; and then

curing said liquid sealant on the egg to form an elastic seal thereon.

21. A method as in claim **13**, wherein said applying step comprises the steps of:

providing a preformed elastic seal member; and then

adhering said preformed elastic seal member to said egg.

22. A method as in claim **13**, wherein said seal contains an antibacterial agent.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,339,766

DATED : 23 August 1994

INVENTOR(S) : Patricia V. Phelps, Julius K. Tyczkowski, Toni O. O'Connell, Ann B. Gore

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In the drawings, Sheet 1, Figures 1-3, should appear as per the attached sheet of drawings.

Signed and Sealed this

Twenty-second Day of November, 1994

Attest:

Attesting Officer

BRUCE LEHMAN

Commissioner of Patents and Trademarks

FIG. 1

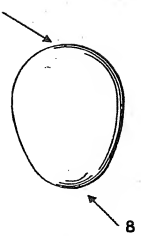


FIG. 2

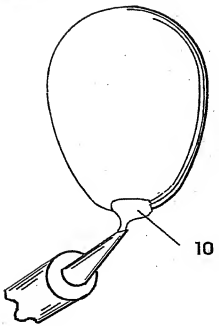
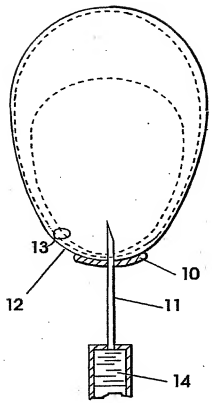


FIG. 3



US005538733A

United States Patent [19]

Emery et al.

[11] Patent Number: 5,538,733

[45] Date of Patent: Jul. 23, 1996

[54] METHOD OF PRIMING AN IMMUNE RESPONSE IN A ONE-DAY OLD ANIMAL

[75] Inventors: Daryll A. Emery; Darren E. Straub; Richard Huisinga, all of Willmar, Minn.

[73] Assignee: Willmar Poultry Company, Inc., Willmar, Minn.

[21] Appl. No.: 272,116

[22] Filed: Jul. 7, 1994

[51] Int. Cl.[6] A61K 39/00; A61K 39/02; A61K 39/12

[52] U.S. Cl. 424/422; 424/423; 424/426; 424/184.1; 424/131.1; 424/204.1; 424/206.1; 424/211.1; 424/214.1; 424/216.1; 424/213.1; 424/220.1; 424/222.1; 424/229.1; 424/207.1; 424/234.1; 424/237.1; 424/239.1; 424/240.1; 424/241.1; 424/257.1; 424/258.1; 424/259.1; 424/274.1

[58] Field of Search 424/450, 422–426, 424/84, 489

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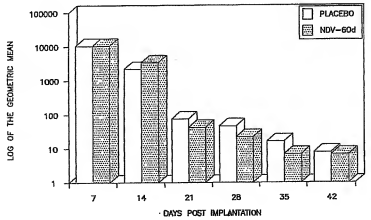

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Primary Examiner—Gollamudi S. Kishore
Attorney, Agent, or Firm—Merchant, Gould, Smith Edell, Welter & Schmidt

[57] **ABSTRACT**

The invention provides a method of priming an immune response in the presence of maternal antibody in a young animal by administering to a 1–90 day old animal, a biocompatible and non-toxic solid phase implant containing an immunogenic agent. A preferred implant according to the invention is one that becomes gradually disintegrated in situ the animal. The implant provides for extended sustained delivery of the immunogenic agent into surrounding tissue fluids in the presence of circulating maternal antibodies to provide a priming dose of the immunogenic agent to stimulate substantially immediate antibody production for active immunity against a pathogen when passive protection is no longer provided by circulating maternal antibodies.

18 Claims, 8 Drawing Sheets

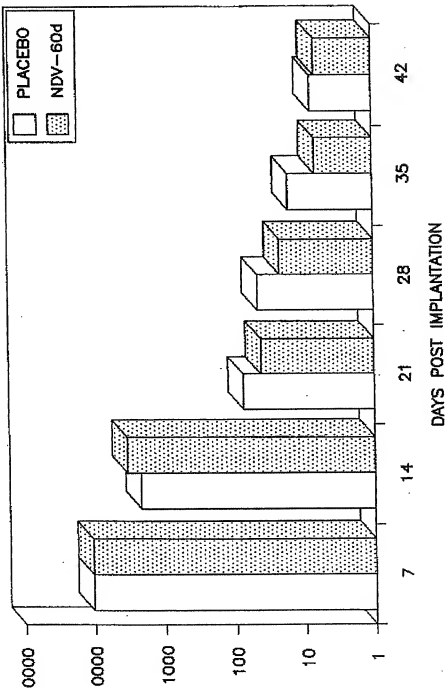

PLACEBO
NDV-60d
100000
10000
1000
100
10
1
LOG OF THE GEOMETRIC MEAN
7
14
21
28
35
42
DAYS POST IMPLANTATION

FIG. 1A

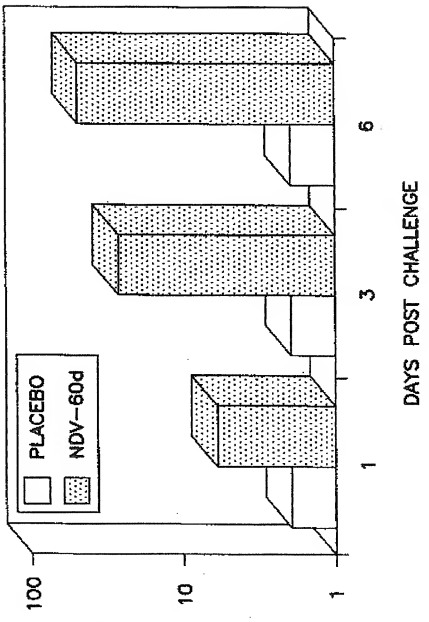
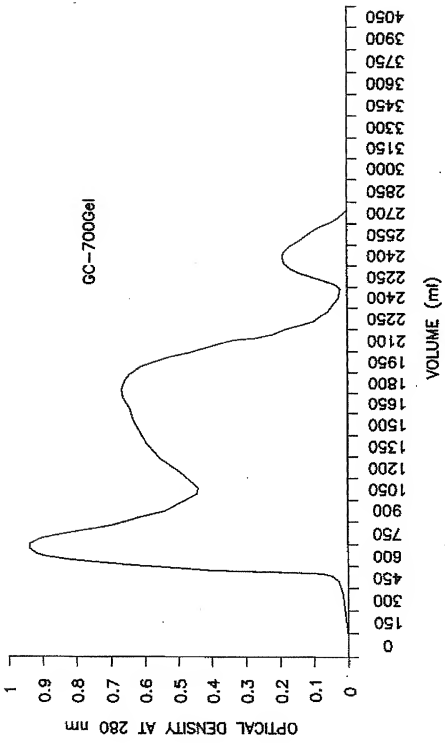

PLACEBO
NDV-60d
100
10
1
LOG OF THE GEOMETRIC MEAN
1
3
6
DAYS POST CHALLENGE

FIG. 1B


GC-700Gel
OPTICAL DENSITY AT 280 nm
VOLUME (ml)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
150
300
450
600
750
900
1050
1200
1350
1500
1650
1800
1950
2100
2250
2400
2250
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2700
2850
3000
3150
3300
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3600
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3900
4050

FIG. 2

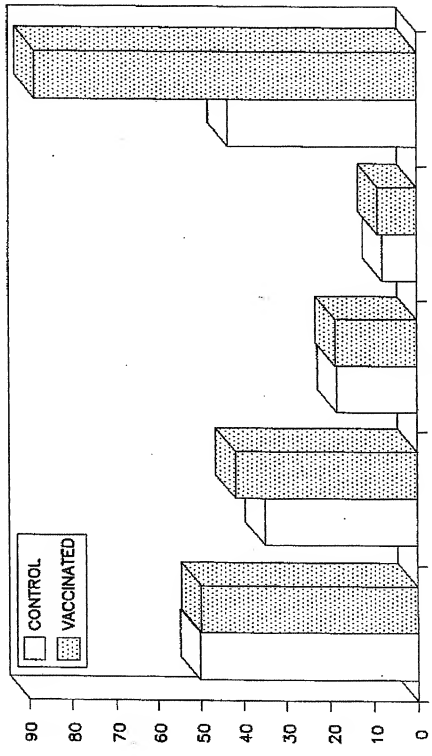
CONTROL
VACCINATED
SRP TITER X 1000
0
10
20
30
40
50
60
70
80
90
7
14
21
28
49
DAYS POST IMPLANTATION

FIG. 3

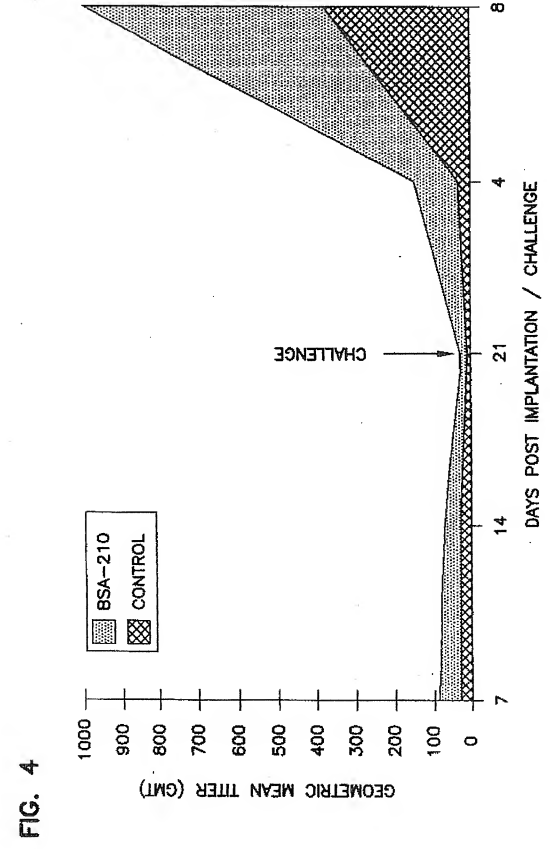
GEOMETRIC MEAN TITER (GMT)
1000
900
800
700
600
500
400
300
200
100
0
BSA-210
CONTROL
CHALLENGE
7
14
21
4
8
DAYS POST IMPLANTATION / CHALLENGE

FIG. 4

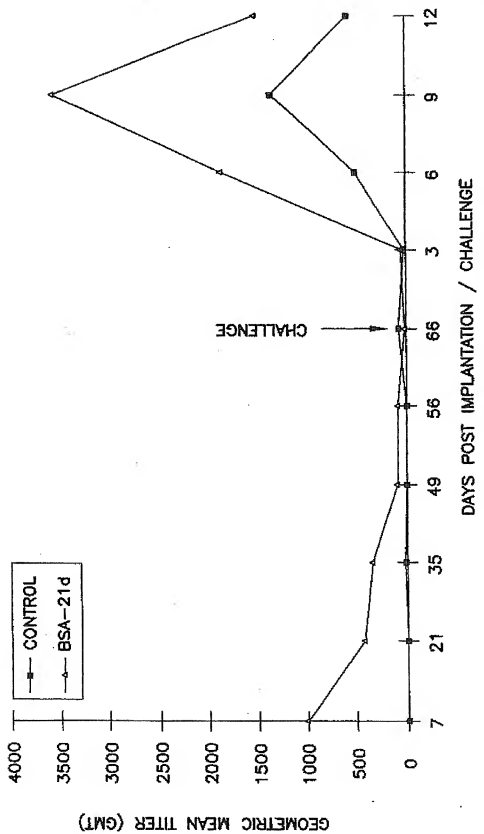
CONTROL
BSA-21d
CHALLENGE
GEOMETRIC MEAN TITER (GMT)
DAYS POST IMPLANTATION / CHALLENGE
4000
3500
3000
2500
2000
1500
1000
500
0
7
21
35
49
56
66
3
6
9
12

FIG. 5

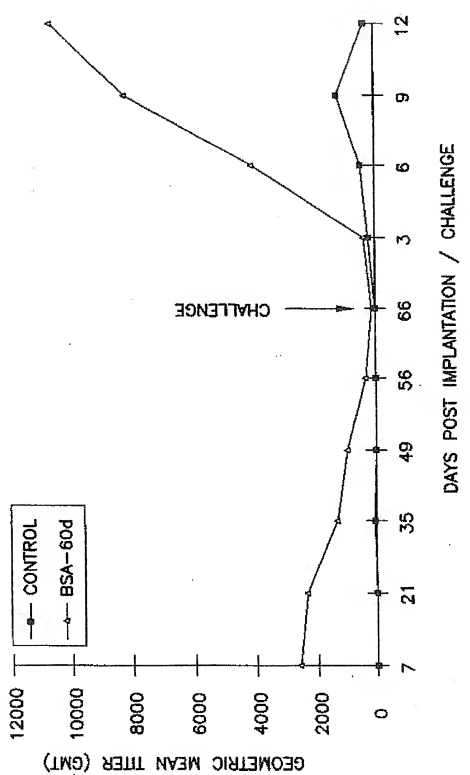
GEOMETRIC MEAN TITER (GMT)
12000
10000
8000
6000
4000
2000
0
CONTROL
BSA-60d
CHALLENGE
7
21
35
49
56
66
3
6
9
12
DAYS POST IMPLANTATION / CHALLENGE

FIG. 6

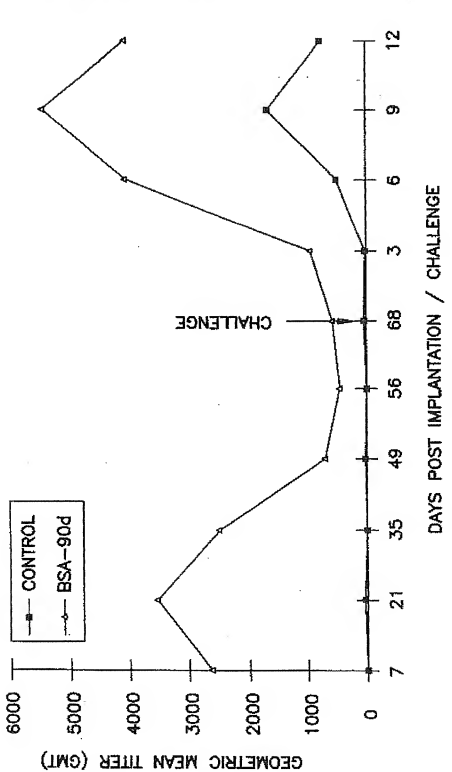
GEOMETRIC MEAN TITER (GMT)
6000
5000
4000
3000
2000
1000
0
CONTROL
BSA-90d
CHALLENGE
7
21
35
49
56
68
3
6
9
12
DAYS POST IMPLANTATION / CHALLENGE

FIG. 7

METHOD OF PRIMING AN IMMUNE RESPONSE IN A ONE-DAY OLD ANIMAL

BACKGROUND OF INVENTION

To properly protect a herd or flock of animals from bacterial and viral infections, it is important that the animals maintain a level of immunity against a pathogen at the point when effective passive immunity from circulating maternal antibodies is lost. In mammals, a fetus receives maternally-synthesized antibodies while in utero which confers passive protection to the fetus. In the avian species, immunity is transferred from the hen via the egg, and the progeny in the first few months of life are protected against toxins, viruses and pathogenic bacteria. The levels of these maternal antibodies gradually decline as the newborn begins to synthesize its own antibodies.

The first exposure of an animal to a particular immunogen triggers a priming response. After initial contact with an immunogen, there is a latent period before antibody to that immunogen is detectable in the serum, generally about 1–2 weeks, during which time T and B cells make contact with the immunogen, proliferate, differentiate, and secrete antibody which then increases exponentially in the serum of the animal, reaches a steady state and then, as the immune response shuts down, decreases in concentration in the serum. This priming contact with an immunogen leaves the immunized animal with a cellular memory of the contact such that, upon a second, later contact with the immunogen, a secondary or anamnestic (memory) immune response is automatically triggered in which the lag phase is considerably shorter and the antibody appears much faster and at a higher concentration in the serum. This capacity to make a secondary response provides an advantage to an animal that survives the first contact with an invading pathogen.

Maternal antibody, while capable of providing passive protection to the neonate against a variety of infectious agents or their toxins, may also interfere with the animal's active response to an immunogen by reacting with and tying up the immunogen. As a result, administration of a priming dose of an immunogen to stimulate active immunity in the newborn must be delayed until the level of circulating maternal antibodies has decreased.

To provide continuous coverage so that immunity is maintained after the disappearance of maternal antibodies, the herd may be immunized en masse when all animals have lost passive immunity. A drawback of this approach is that there will be a certain percentage of animals who lose immunity before the rest of the group and are therefore vulnerable during the interim. Another approach is to vaccinate an animal with an immunogen repeatedly from day one until they are found capable of responding, but the stress on the animal and expense for the breeder prohibits this tactic. Yet another approach is to administer to a young animal, a priming dose of an immunogen in a preparation that will present the immunogen in a slowly dissipating material. Examples of such preparations are an injectable water-in-oil emulsion containing live virus, a suspension of an antigen in Freund's complete adjuvant or phosphatidylcholine (egg-lecithin)- or cholestgrol-based liposomes containing an entrapped immunogen. However, such preparations do not provide adequate long-term delivery of an immunogen and may cause adverse reactions such as granuloma formation. There is also the risk of the person administering the injection accidentally injecting themselves with the preparation, resulting in an adverse reaction from the injected ingredients. For example, injecting mineral oil into a human finger, or other tissue or body part, will cause severe reaction and may result in the loss of tissue or worse.

Therefore, it is an object of the invention to provide an effective method for priming the immune system of a young animal to achieve a virtually immediate active immune response to infection by a pathogenic organism at the point where such protection is no longer provided by maternal antibodies, without irritation or undue stress caused to the animal. Another object is to provide an effective method for immunizing a large number of animals.

SUMMARY OF THE INVENTION

These and other objects are achieved by the present invention which is directed to a method of priming an immune response in a young animal by administering a biocompatible and non-toxic solid phase implant containing an immunogenic agent. The implant is administered to the animal at about 1–90 days of age in the presence of circulating maternal antibodies, and provides extended sustained delivery of a priming dose of the immunogenic agent into surrounding tissue fluids in the presence of circulating maternal antibodies. A preferred application is the administration of the implant to a one-day old animal.

Advantageously, the present method provides a system for delivering a priming dose of an immunogen to a young animal in the presence of circulating maternal antibodies over an extended time so that the animal will produce a secondary active immune response substantially immediately upon contact with the immunogen when passive protection is no longer provided by circulating maternal antibodies. The priming dose of the immunogen released from the implant is effective to elicit a secondary immune response in the animal to increase the anti-immunogen antibodies to an antibody titer of about 10–1000, or an about 5–100 fold increase in antibody titers.

A preferred implant matrix is made of a biocompatible, biodegradable, bioabsorbable and/or bioerodible polymeric material that will become gradually disintegrated by the animal's system through enzymatic, chemical and/or cellular hydrolytic action, and release the immunogen for sustained delivery into surrounding tissue fluids over an about 1–90 day period. The implant may be formulated, for example, from cholesterol, cellulosic polymers, polylactide, polycaprolactone, polyglycolide or other like polymers or copolymers thereof. The implant may include immunostimulants such as aluminum hydroxide, muramyldipeptide, lipophilic amines, saponins, Freund's incomplete adjuvant (FIA), polymeric adjuvants, among other adjuvants. The implant may also contain an immunomodulator such as a cytokine, complex carbohydrate, and the like to enhance or modulate the immune response, and other additives as desired, such as preservatives, buffering agents, and the like.

The immunogen may be any substance that is structurally and/or functionally capable of stimulating an immune response in an animal, and which may be incorporated into and subsequently released from the implant matrix into surrounding tissue fluids. The immunogen may be in the form of a whole bacterial cell or viral particle, or an isolated and/or substantially pure immunogenic molecule derived therefrom such as a cell surface glycoprotein, sphingolipid and the like; a toxin, allergen, hormone, or anti-idiotype; or a synthetic polypeptide, and the like.

The implant may be administered to the animal by subcutaneous or intermuscular implantation, preferably through

the use of an injection gun as known and used in the art, which accommodates administering the implant to a large number of animals within a short period of time. Once implanted, the matrix provides for the sustained release of the incorporated immunogenic agent over an about 1–90 day period into the surrounding tissue fluids of the animal.

Advantageously, the present method of priming an animal by administering an immunogen-containing implant into the animal provides for enhanced immune levels at the point of transition between maternal antibodies and active production of antibodies. The present method provides a means for immunizing a young animal, particularly an animal at one day of age, easily and with a minimal amount of stress placed on the animal, and without adverse reactions such as granuloma formation, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B are graphic depictions of the immune response to New Castle Disease (NDV) virus in turkey poults administered a 60-day release, cholesterol-based, metabolizable implant at 1-day of age. FIG. 1A shows the serological profile of maternal antibody to NDV between placebo and vaccinated birds (placebo=□; NDV-60 day=■). FIG. 1B sbows the immune response to NDV after challenge using a 60-day release metabolizable implant (placebo=□; NDV-60 day=■).

FIG. 2 is a graphic depiction of the elution profile of concentrated, solubilized siderophore receptor proteins isolated from *Pasteurella multocida* serotype A:3 (ATCC 15742).

FIG. 3 is a graphic depiction of the immune response to *P. multocida* siderophore receptor proteins in turkey poults administered a 60-day release, cholesterol-based, metabolizable implant at 1-day of age (control=□; vaccinated=■).

FIG. 4 is a graphic depiction of the immine response to Bovine Serum Albumin (BSA) in turkey poults administered a 21-day release, cholesterol-based, metabolizable implant at 1-day of age (BSA-21 day=□; control=■).

FIG. 5 is a graphic depiction of the immune response to BSA in turkey poults administered a 21-day release, cholesterol-based metabolizable implant at 2 weeks of age (BSA-21 day=—Δ—; control=—■—).

FIG. 6 is a graphic depiction of the immune response to BSA in turkey poults administered a 60-day release, cholesterol-based metabolizable implant at 2 weeks of age (BSA-60 day=—Δ—; control=—■—).

FIG. 7 is a graphic depiction of the immune response to BSA in turkey poults administered a 90-day release, cholesterol-based metabolizable implant at 2 weeks of age (BSA-90 day=—Δ—; control=—■—).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for priming an immune response in a 1–90 day old animal, in the presence of circulating maternal antibodies, by employing a biocompatible implant containing an immunogenic agent. According to that method, a continuous amount of the immunogenic agent effective to provide a priming dose of the immunogen is maintained in the circulatory system of the animal by its release from the implant matrix into the tissue fluids of the young animal. The continuous presence of a priming dose of the immunogen provides an effective way of priming a young animal so that a secondary immune response to a pathogenic infection is stimulated substantially immediately when passive protection by maternal antibodies against the pathogen is no longer effective.

As used berein, the term "substantially purc" means that the immunogen has been separated from its natural association with other constituents such as proteins, lipids, and other like substances and elements of a bacterial cell, viral particle and the like. The term "biodegradable" means that the implant matrix will degrade over time by enzymatic or bydrolytic action, or other like mechanism in the animal's body. By "biocrodible," it is meant that the implant will erode or degrade over time by the contact with surrounding tissue fluids, cellular activity and the like. By "bioabsorbable," it is meant that the implant matrix will break down and be absorbed by a cell, tissue, and the like, within the animal's body. By "biocompatible," it is meant that the implant matrix does not cause substantial tissue irritation or necrosis at the implant site.

Implant matrix. The implant administered according to the method are those known and used in the art that comprise a biocompatible, non-toxic material. Implants useful in the method have a matrix structure that will allow for incorporation of the immunogenic agent and release of the agent at a desired rate.

A preferred implant is made of a biocompatible solid pbase polymeric matrix that is bioabsorbable, biodegradable, and/or bioerodible in the body of the animal, and will deliver an immunogen into tissue fluids over an extended period without irritation and adverse effects to the animal. Such polymers allow for the breakdown of the implant during therapy, generally to monomeric subunits that are biocompatible with the surrounding tissue.

Examples of useful polymers for forming a biodegradable, bioabsorbable implant include polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone, polyanhydride, polyamides, or copolymer thereof such as copolymers of polyamides and polyesters, copolymers of PLA and PGA, and the like. In general, the in vivo life of an implant formulated with such polymers will depend at least in part, on the molecular weight and degree of crosslinking of the polymer in the matrix. Formulations for such matrices are known in the art, as disclosed for example, in U.S. Pat. No. 3,887,699 to Yolles.

Other implants useful in the method include biodegradable, metabolizable, cholesterol-based pellets that provide for slow release of bioactive substances. Cholesterol-based implant matrices are commercially-available, for example, as 21-, 60-, and 90-day implants from Innovative Researcb, Toledo, Ohio. Other cholesterol-based implants bave been described for slow release of biotin and other micronutrients, and proteins, polynucleotides, polysacharrides, for example, U.S. Pat. No. 4,452,775 to Kent; and U.S. Pat. No. 4,326,523 to Wolfrom. Also useful are implants having a peptide/polymer matrix, for example, tyrosine dipeptides and polymers as described in U.S. Pat. No. 4,863,735 to Kohn, and Kohn et al., *J. Immunol. Methods* 95:31–38 (1986), that will degrade to form a product having adjuvant activity for the antigen or other bioactive compound incorporated into the matrix.

Also useful according to the invention is a time-delayed implant, known and used in the art, which upon implantation, will substantially maintain integrity of the matrix for a desired length of time. Preferably, the matrix will remain intact for up to about 3 weeks, or after the level of maternal antibody has significantly declined, at which time the antigen is released from the matrix.

Although not preferred, an implant made of a non-erodible, synthetic polymer, as for example, a hydrogel, a high density polyethylene, or ethylene-vinyl acetate copolymer (EVAC), may be used according to the method for slow delivery of the immunogen. Such implant matrices are described, for example, by Niemi et al., *Laboratory Animal Science* 35:609 (1985) (EVAC); Radomsky et al., *Biol. Reprod.* 47:133–140 (1992) (EVAC); U.S. Pat. No. 5,114,719 to Sabel (EVAC et al.); and U.S. Pat. No. 3,975,350 to Hudgin (hydrogel carrier). However, such implants do not naturally degrade in the body and require surgical removal after the immunogen has been delivered into the body of the animal. In addition, EVAC implants have been shown to cause irritation resulting in necrosis at the implant site. Hydrogels are a polymeric material that swell but will not dissolve in water, and have a structural rigidity imparted by crosslinking agents, as for example, polyhydroxyalkyl methacrylates (P-HEMA), polyacrylamide, polymethacrylamide, polyvinyl pyrrolidone, polyvinyl alcohol (PVA), among others. Low molecular weight substances tend to diffuse relatively quickly through a hydrogel matrix which may be a disadvantage for controlled delivery.

Immunogenic Agent. The method provides sustained and/or timed release of an immunogenic agent from the implant matrix. The immunogenic agent is a substance that is effective in stimulating production of antibodies with specific activity against the immunogenic agent and the pathogenic organism against which the animal is being immunized. The immunogen has a chemical structure which provides for its incorporation into the implant matrix such that over time, the immunogen will be released from the matrix into the adjacent tissue fluids, preferably at a controlled rate. The immunogenic agent may be any antigenic substance that is capable of stimulating an immune response in the animal being treated. The implant may be formulated to include a single immunogen or a mixture of immunogens for immunizing the animal against one or more diseases and/or infections. Preferably, the implant contains about 25–5000 μg/mg of the immunogenic agent preferably about 100–2000 μg/mg, preferably about 250–1000 μg/mg.

The immunogen may be derived from a pathogenic organism such as a bacteria, virus, fungi, mold, protozoans, nematodes, or other organism. The immunogen may be in the form, for example, of whole bacterial cells, whole viral particles, immunogenic subunit molecules or secreted substances derived therefrom, isolated nucleic acid preferably bound to a carrier protein, and the like. Immunogens may be prepared according to conventional isolation and purification methods, and/or by gene expression according to recombinant DNA techniques to make and express a gene encoding all or part of an antigenic peptide chain in an appropriate vector (i.e., vaccinia virus recombinants). Such immunogenic subunits include, for example, subunit vaccine polypeptides, cell membrane glycoproteins, polysaccharides, sphingolipids, lipopolysaccharides, and the like. See, for example, Harlow and Lane, *Antibodies, A Laboratory Manual*, generally and Chapter 5, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, N.Y. (1988); Male et al., *Advanced Immunology*, generally and pages 14.1–14.15, J. B. Lippincott Co., Philadelphia, Pa. (1991); and Roitt et al., *Immunology*, generally and pages 16.1–17.21, J. B. Lippincott Co., Philadelphia, Pa. (1989).

Also useful are immunogenic synthetic peptides that mimic antigenic peptide sequences. Such immunogens may be synthesized using a solid-phase technique as described, for example, in R. B. Merrifield, *Science* 85: 2149–2154 (1963), purified, and optionally coupled to a carrier protein such as muramyldipeptide (MDP), bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), and the like, using a bifunctional coupling agent such as glutaraldehyde, and the like.

Other useful immunogens are purified, secreted antigen virulence factors, such as toxins, cytotoxins, and the like. Toxin antigens which are detoxified by modifying (toxoids), preferably administered in combination with an adjuvant such as aluminum hydroxide, may be used to stimulate the formation of toxin-neutralizing antibodies. Examples of toxins that may be used as an immunogen include bacterial endotoxins and exotoxins, enterotoxins including heat-labile enterotoxins (LT), heat stable enterotoxins (ST), verotoxin (VT), and the like. Bacterial exotoxin immunogens are secreted into the surrounding medium, and include, for example, diphtheria toxin (*Corynebacterium diphtheriae*), tetanus toxin (*Clostridium tetani*), enterotoxins secreted by *Staphylococcus aureus*, botulinus toxins (*Clostridium botulinum*); and toxins produced by algae such as neurotoxins; and the like. Heat-stable endotoxins, released by autolysis of the bacteria, include, for example, cholera toxins released from the gram negative *Vibrio cholerae*, colicins produced by intestinal bacteria such as *E. coli* (bacteriocin). In brief, toxoids may be prepared, for example, by culturing the bacteria and, after the required growth is attained, filtering the culture to obtain a filtrate containing the toxin (exotoxin), precipitating the toxin from the filtrate using, for example, a concentrated salt solution, washing the precipitated toxin and purifying it by dialysis, and then detoxifying the toxin with formaldebyde. An implant may contain the detoxified toxin (toxoid) plain or with an adjuvant such as alum, aluminum hydroxide or aluminum phosphate. Other toxins may be cell associated and released by rupturing the cell wall by sonication or French pressure, and the like, and the ruptured material then centrifuged and treated as described above.

Another useful immunogen is an allergen such as pollen, danders, mold spores, mycelial fragments, epidermals, and the like, capable of stimulating IgE production.

Also useful is a hapten, or low molecular weight substance such as an antibiotic, drug, peptide, among others, which when conjugated to an immunogenic carrier such as a protein, carbohydrate, lipid, or other like carrier, for example, BSA and KLH, using a bifunctional coupling agent, will induce an immune response directed against the parts of the conjugate. The preparation of hapten immunogens is described, for example, in Harlow and Lane, *Antibodies: A Laboratory Manual*, pages 72–87, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, N.Y. (1988).

Another immunogen is an anti-idiotype antibody that reacts with the antigen binding site of the idiotype antibody, and structurally mimics the epitope, i.e., contains the internal image of the epitope. The anti-idiotype may be used as an immunogen to induce antibodies against the original epitope. Briefly, anti-idiotypic antibodies may be induced by injecting an antigen into a rabbit and allowing the immune system to produce immunoglobulins. These immunoglobulins are then harvested and injected into a second animal such as a domestic hen. The hen then mounts an immune response to the foreign antibodies with the production of immunoglobulins that mimic the original antigen. Anti-idiotype antibodies may then be isolated from the yolks of eggs laid by the hen by methods known in the art, and used as an original immunogen. A useful method for isolating antibodies from egg yolks is according to the method described in U.S. patent application Ser. No. 08/118,514,

now U.S. Pat. No. 5,420,253, entitled "Method for Purifying Egg Yolk Immunoglobulins." For further discussion of anti-idiotypes as vaccines, see, for example, Bhogal et al., *Crit. Rev. Poultry Biol.* 3: 53–68 (1991); and Bhogal et al., Infection and Immunity 56: 1113–1119 (1988).

The immunogen may also be derived from RNA or DNA viruses. Examples of such viruses include New Castle disease virus (NCDV), hemorrhagic enteritidis virus (HEV), infectious rhinotracheitis virus (IRRV), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus BRSV), hog cholera virus (HCV), equine encephalomyelitis virus (EEV), canine distemper virus, fowl pox virus, rabies virus, avian leukosis virus, turkey virus, infectious bursal disease virus, infectious bronchitis virus, avian influenza virus, avian encephalomyeliis virus, among others. Techniques for the preparation of virus-derived immunogens are known in the art, and described, for example, in Ulmer et al., *Science* 259:1745 (1993); Male et al., *Advanced Immunology*, pages 14.1–14.15, J. B. Lippincott Co., Philadelphia, Pa. (1989).

Immunogen may also be derived from bacteria such as *Escherichia coli;* Salmonella spp. such as *Salmonella agona, Salmonella blockley, Salmonella enteriditis, Salmonella hadar, Salmonella heidelberg, Salmonella montevideo, Salmonella senftenberg, Salmonella cholerasuis;* Pasteurella spp. such as *Pasteurella haemolytica* and *Pasteurella multocida;* Pseudomonas spp. such as *Pseudomonas aeruginosa;* Klebsiella spp. such as *Klebsiella pneumoniae;* Actinobacillus spp. such as *A. pleuropneumoniae,* Haemophilus spp. such as *H. parasuis;* Streptococcus spp. such as *S. suis;* Bordetella spp. such as *B. bronchiseptica, B. avium;* among other gram negative bacteria. Examples of gram positive bacteria from which useful immunogens may be derived include Staphylococcus spp., Streptococcus spp., Erysipelothrix spp., Clostridium spp., among others.

The immunogenic agent may also be derived from a fungi or mold such as *Aspergillus flayus, Aspergillus fumigatis,* Penicillium spp., Fusarium spp., Candida spp. such as C. Trichophyton spp., Rhizopus spp., and other fungi and molds; protozoa such as Treponema, Toxoplasma, Cryptococcus, Coccidia, Histomoniasis, Hexamitiasis, Giardia, among others; spirochetes such as Borrelia spp; nematodes including Ascaris spp., Trichinella spp., and the like, helminthes such as flukes, tapeworms, among others; and other like pathogenic organisms. Methods for preparing immunogens derived from fungi, molds, protozoa, nematodes, and helminthes are known in the art, and described, for example, by Douglas R. Yearout, 1988 *Proc. Assoc. of Avian Veterinarians,* pages 139–144 (1988) (aspergillus).

A useful immunogenic agent is a siderophore receptor protein (SRP) which is a protein or suhunit immunogenic peptide of the protein derived from the outer membrane of a gram-negative hacteria or other pathogenic organism such as a mold or fungus, that will hind a siderophore, or iron-binding protein. Implants containing an SRP immunogen can be administered to elicit an immune response in an animal with the production of anti-SRP antibodies that react with SRPs of the same organism from which the SRP immunogen was derived or cross-react with a SRP of a pathogenic organism of a different strain, species or genus. Examples of siderophore receptor proteins are hydroxamates and phenolates such as aerobactin, enterochelin, citrate, multocidin, ferrichrome, coprogen, mycohactin, and the like, or an immunogenic fragment thereof, that will stimulate production of anti-SRP antibodies.

Siderophore receptor proteins having a molecular weight of about 72–96 kDa, as determined by SDS-PAGE, have heen isolated from *E. coli,* Salmonella spp., Pasteurella spp., Pseudomonas spp., and Klebsiella spp. An implant may he formulated to contain, for example, one or more SRPs derived from a serotype of *E. coli* such as *E. coli* serotype 01a, 02a and/or 078 (American Type Culture Collection (ATCC), Bethesda, Md., ATCC Accession no. 55652, on Jan. 3, 1995), the SRP having a molecular weight of about 89 kDa, 84 kDa, 78 kDa or 72 kDa (SDS-PAGE), and effective to stimulate antihodies immunoreactive with the *E. coli* from which the protein is derived and a second gram-negative bacteria such as a species of Salmonella, Pseudomonas, Klebsiella, and/or Pasteurella. Another useful implant may include one or more SRPs derived from a species of Pasteurella, having a molecular weight of about 96 kDa, 84 kDa or 80 kDa; and/or a species of Salmonella having a molecular weight of about 89 kDa, 81 kDa or 72 kDa. In brief, to obtain SRPs for use as immunogens, the SRP-producing organism is cultured in a medium that lacks iron or includes an iron chelating agent and, after harvesting, the SRPs are separated from the organism's outer membrane and purified by treating with an anionic detergent, preferably sodium dodecyl sulfate (SDS) and under non-reducing conditions, as described, for example, in co-pending U.S. patent application Ser. No. 08/194,094 entitled "Active Immunization using a Siderophore Protein."

Yet another implant useful in the present method is one that is formulated to contain vasoactive intestinal peptide (VIP), or an immunogenic fragment thereof. VIP is an about 28 amino acid, synthetically produced peptide, that has been shown to control the release of prolactin in a hen. This, in turn, inhihits nesting behavior, or broodiness, of a hen. VIP is commercially available, for example, from Peninsula Laboratory, Inc., Belmont, Calif. Active immunization by injection with neutralized, endogenous VIP in Freund's incomplete adjuvant has shown a decrease in circulating prolactin and elimination of nesting behavior in hens. However, such injections induce severe granuloma formation and cause added stress to the hen. Surprisingly, the present method of administering a VIP preparation formulated as a metabolizable implant for slow release into the hen has been shown to effectively immunize hens without inducing granuloma formation or other adverse reactions.

In brief, a VIP implant may be prepared by linking the VIP to a carrier protein such as KLH or BSA by means of a bifunctional coupling agent such as m-maleimidobenzoyl-N-hydroxysuccinimide (MBS), carhodiimide, his-diasotized bensidene (BDB), among others, as described, for example, in Francis et al., *J. Gen. Virol.,* 66:2347–2354 (1985). Preferably, the VIP-KLH conjugate is incorporated into a metabolizable implant material, preferably a cholesterol-based material, which provides sustained release of an effective amount of VIP from the implant matrix into the tissue fluids of the hen to suppress the release of prolactin in the bird. This, in turn, maintains egg production by inhibiting nesting behavior in the hen. The VIP implant is formulated to contain about 25–5000 μg VIP per mg of the implant matrix, preferably about 25–1000 μg/mg, and to provide a rate of release of VIP from the matrix of about 0.01–2 μg/kg weight of the hen per day, preferably about 0.15–1 μg/kg/day. The hen may be treated to receive VIP over an about 1–210 day period, using one or more VIP-containing implants. A preferred implant contains about 16 μg/mg, and provides a release of about 2.5–3 μg/day in a 30 lh (13.6 kg) hen.

Adjuvants. The implant may include physiologically-acceptable adjuvants as desired to enhance the immune response in the animal. Such adjuvants include, for example,

aluminum hydroxide, aluminum phosphate, Freund's incomplete adjuvant (FIA), liposomes, immunostimulating complex (ISCOM), muramyldipeptide (MDP), polysaccharides such as Acemann, and the like. Preferred adjuvants are $Al(OH)_3$ combined with the antigen at about 0.5%, and FIA combined with the immunogen at a ratio of about 1:1. Preferably, where included, the implant matrix comprises about 0.01–10 wt-% of an adjuvant.

Additives. The implant may be formulated with one or more additives to maintain isotonicity, physiological pH and stability, for example, a buffer such as physiological saline (0.85%), phosphate-buffered saline (PBS), citrate buffers, Tris(hydroxymethyl aminomethane (TRIS), Tris-buffered saline and the like, and/or a preservative such as thimersol, formalin, glutaraldehyde, or an antibiotic, for example, neomycin or streptomycin, and the like.

It is also envisioned that the immunogen may be combined with a biocompatible, and optionally synergistic, immunomodulator that cooperatively stimulates antibody production, as for example, recombinant cytokines such as TGF-beta, interferons, activating factors, chemoattractants, interleukins such as IL-1, IL-2, IL-4, IL-5, IL-6, complex carbohydrates such as Acemann (available commercially from Solvay), and other like substances. Immunomodulators are described, for example, in Hudson and Hay, *Practical Immunology*, pages 423–441, Blackwell Scientific Publications, London (1989); and Male et al., *Advanced Immunology*, pages 11.1–11.16, J. B. Lippincott Co., Philadelphia, Pa. (1991).

The matrix may optionally be formulated to include a soluble or insoluble pore-forming agent that will dissipate from the matrix into surrounding tissue fluids causing the formation of pores and/or channels throughout the implant matrix. Examples of such pore-forming agents include sodium chloride and other salts; carboxymethylcellulose, polyethylene glycol and other polymers; starch, glucose and other carbohydrates; amino acids and low molecular weight non-immunogenic proteins and the like. The implant matrix may be formulated to include about 0.05–65 wt-% of a pore-forming agent.

Dosage and Administration. The implant may be used to immunize domestic fowl and other animals such as livestock, horses, companion animals such as cats and dogs, and humans, against infection caused by one or more pathogenic organisms such as a virus, gram-negative bacteria, fungi, mold, and the like. Choice of the particular formulation will depend upon the condition to be treated and the desired release rate, which choice is made by an animal-care professional.

The implant is formulated with an amount of the immunogenic agent effective to provide a desired priming function for the animal's immune system. The "effective amount" of the immunogenic agent included in the matrix is according to the desired release profile, the concentration of immunogen required for a desired priming effect, and the period of time over which the immunogen is to be released. Factors bearing on the vaccine dosage include, for example, the age and weight of the animal. Ultimately, the amount of the immunogen included in the implant is determined by an animal care professional. There is generally no maximum amount of the immunogenic agent that is incorporated into the solid matrix, except for physical limitations that allow the immunogenic agent to be held within the matrix and released in a predetermined manner. Generally, the implant is formulated to contain about 25–5000 μg of the immunogenic agent per mg of the implant matrix, preferably about 100–2000 μg/mg, preferably about 250–1000 μg/mg.

Advantageously, the present method provides for the continuous delivery of an immunogenic agent into the circulatory system of the animal as the level of circulating maternal antibodies diminishes over time and becomes insufficient for providing passive protection against infection by a pathogen. The continuous presence and amount of the immunogenic substance released from the implant matrix provides a priming dose of the antigen effective to stimulate an immune response in the animal in the presence of decreasing maternal antibody titers, substantially immediately upon challenge by a pathogenic bacteria, virus or other organism. In avian species an about 60- to 90-day release implant containing the immunogen will be suitable for delivery of the immunogen, while in mammals, an about 90-day implant is preferred.

The in vivo release rate and extent of release of the immunogenic agent from the solid implant matrix may be effectively controlled and optimized, for example, by varying the matrix formulation according to the desired duration or time interval for maintaining the solid matrix within the implant site, and by varying the type and amount of adjuvants and additives, such as plasticizing agents, and by the size, shape, porosity, solubility and biodegradability of the matrix, among other factors, according to practices known and used in the art. The release of the immunogen from the matrix may also be varied according to the form and solubility of the immunogenic agent in tissue fluids, the distribution of the immunogen within the matrix, among other factors.

The implant is formulated to release a linear dosage amount of the immunogenic agent into the body of the animal over a period of about 1–90 days, preferably about 1–60 days. The release is based on the biodegradability, bioabsorbability and/or bioerodibility of the implant matrix in the body of the animal. For example, a cholesterol-based 60-day release implant pellet (Innovative Research) containing about 25–5000 μg of protein antigen per mg implant matrix, for example, *E. coli* siderophore protein antigen, maintaining a linear release, will release on a daily basis, about 0.4–83 μg/day of protein immunogen. A similar cholesterol-based 21-day implant containing about 5000 μg protein antigen will release about 238 μg/day, and a 90-day implant will release about 56 μg/day. Thus, the implant is formulated to include an amount of an immunogen to provide the desired amount released into the bloodstream over a predetermined time period.

Once implanted, the implant matrix provides for the sustained release of the immunogenic agent into surrounding tissue fluids in the presence of circulating maternal antibodies over the desired time period, preferably disintegrating gradually by the action of the animal's system. The amount of immunogen released from the implant will effectively induce a primary immune response in the animal, so that the animal will respond by the production of antibodies (i.e., secondary immune response) when there is later contact with the immunogen and/or pathogenic organism and the material antibody titer is no longer at a protective level.

Once the animal is primed, a booster is advantageously administered to the animal to stimulate a secondary immune response in the animal. The booster may be in the form of a second sustained-release implant containing the immunogenic agent, an injectable liquid vaccine, a modified live vaccine, a natural exposure to the immunogen/pathogen, or other suitable means. For example, a booster effect in a bird primed with SRPs may come from a natural field exposure with a bacteria that expresses an SRP that will cause a rise in anti-SRP antibody titers. The level of anti-SRP antibodies

then remains elevated throughout the life of the bird to protect the animal against lethal challenge by the pathogenic organism. An animal may also be boosted to stimulate a secondary response by injection with an immunogen, for example, a modified live vaccine, at a time after implantation. For example, a 21-day implant containing about 50–500 μg protein antigen and having a release rate of about 8.3 μg/day, may be administered to a one-day old turkey poult, and then a booster injection containing about 500–1500 μg protein may be administered to the poult after the expiration date of the implant (i.e., after 21 days), for example, at about 28–42 days after implantation, to stimulate a secondary immune response. The booster may also be provided from the continued release of the immunogen from the implant used for priming the animal, in which case, after the animal has been primed and becomes immunologically mature and decreasing maternal antibody titers no longer provide effective protection, the continued release of the immunogen from the implant may induce active production of antibodies (i.e., secondary response) in the animal.

The amount of immunogen released from the implant to provide a priming response is effective to induce a secondary immune response in the animal upon delivery of the booster immunogen and/or upon challenge by a pathogenic organism resulting in an about 5–50 times higher antibody titer over pre-challenge antibody titers, as measured by Enzyme Linked Immunosorbent Assay (ELISA) assay, more preferably an about 25–100 fold increase, within about 12–48 hours of receiving the booster and/or challenge, preferably within about 6–24 hours. Preferably, the active antibody titer after the booster or post-challenge, is about 10–1000, preferably about 50–500.

In a preferred method, the serological profile of maternal antibody to a particular immunogen is monitored by flock profiling the vaccinated animals by immunoassay, preferably using a direct ELISA as known and used in the art, for example, a commercially-available ELISA kit for the antigen being assayed. The booster is advantageously administered when the maternal antibody titers have declined to a level at which there is inadequate protection provided against infection by a pathogenic organism, for example, to a titer about 5–10% of peak measurement (i.e., an antibody titer of about 50–100 following a peak titer of 1000). The animal is then boosted to elicit a secondary immune response which is then monitored by testing blood samples by immunoassay. For example, the primary immune response to siderophore receptor protein immunogen(s) (SRPs) can be monitored by vaccinating a group of birds at about 3-weeks of age and testing blood samples at about 5-day intervals for about 20 days. At twenty days post-implantation, birds may then be intramuscularly boosted with *E. coli* 078, a gram-negative bacteria that expresses four SRPs on its surface. The birds that have been vaccinated against the proteins will then mount a secondary immune response to the proteins. This response can be monitored by taking blood samples from the birds at about 2–4 day intervals and determining the antibody response by ELISA using SRPs as the capture molecule to react with antibodies specific for SRP.

To determine whether the secondary antibody response is protective, a quantitative clearance may be performed by estimating the number of *E. coli* in organs of the animal. For example, at 24-hour intervals after challenge, the liver and spleens may be aseptically removed from a limited number of animals in the group (vaccinated and non-vaccinated), macerated, and then standard plate counts performed to enumerate the number of bacteria per gram of tissue. The data from the vaccinated birds are compared to non-vaccinated birds, and extrapolated to the remaining animals in the group.

Administration of the implant is ultimately done under the wisdom and protocol of a veterinarian or other animal-care professional. The implant is advantageously and preferably subcutaneously implanted in a 1-day old animal. The implant may also be administered to a young animal up to about 90 days of age, preferably about 1–60 days of age. Implantation is achieved by a suitable method known and used in art, for example, by surgical incision, or preferably by the use of a commercial injection gun.

The invention will be further described by reference to the following detailed examples, wherein the methodologies are as described below. These examples are not meant to limit the scope of the invention that has been set forth in the foregoing description. Variation within the concepts of the invention are apparent to those skilled in the art. The disclosures of the cited references, patents and co-pending patent applications throughout the application are incorporated by reference herein.

EXAMPLE 1

Priming a 1-day Old Poult Against Infection by New Castle Disease Virus (NDV)

Forty 1-day old turkey poults were each administered a 60-day release, cholesterol-based, metabolizable implant (10 mg; Innovative Research, Toledo, Ohio) by subcutaneous injection using an injection gun. Twenty of the poults, i.e. test birds, were implanted with an implant loaded with 10,000 virus particles/implant of New Castle Disease (NCD) virus (LaSota strain) (Solvay Animal Heath, Mendota Heights, Minn.). The stock virus was resuspended in buffered saline and 0.6 ml of β propiolactone (Sigma) was added to inactivate the virus. The killed stock suspension was diluted to 10,000 virus particles and lyophilized with the implant matrix to provide 10,000 virus particles/implant. Twenty of the poults, i.e. control birds, received a placebo implant.

The maternal antibody to NDV was monitored using an NDV-ELISA (commercially available from Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The serological response was monitored at 7-day intervals through 42 days post-implantation. The results are shown in FIG. 1A. At 60 days following implantation, the birds were challenged by intravenously injecting 1-ml of a solution of killed NDV virus at 10,000 virus particles/bird in physiological saline. The serological response to challenge was monitored at 1, 3, and 6 days post-challenge using the above assay.

As sbown in FIG. 1B, all of the test birds implanted with the immunogen-containing implant sero-converted, and sbowed a significant secondary immune response after challenge as compared to the non-vaccinated controls.

EXAMPLE 2

Priming a 1-day Old Poult Against Infection by Gram-negative Bacteria

A 60-day cholesterol-based matrix of 10 mg, as described in Example 1, was prepared with siderophore receptor proteins derived from *Pasteurella multocida* (turkey isolate; ATCC P-1059) by the following method, as described in co-pending patent application Ser. No. 08/194,040.

P. multocida serotype A:3 (700 ml at 10^8 colonies/ml) was inoculated into a Virtis bench-top fermenter (Virtis, Inc., Gardiner, N.Y.), charged with 20-L of brain-heart infusion (BHI, Difco Laboratories, Detroit, Mich.) containing 50 μgrams/ml of dipyridyl (Sigma Chemical Co., St. Louis, Mo.) at 41° C. This isolate has been shown to produce three siderophore receptor proteins for (M.W. 96 kDa, 84 kDa, 80 kDa) under iron-restrictive conditions. The pH was held constant at 7.4 by automatic titration with 5N NaOH. The fermenter was stirred at 400 rpm. The culture was grown continuously for 18 hours after which the bacteria were removed by continuous-flow centrifugation at 20,000×g at 4° C. using a Beckman (Model 22-21M) centrifuge (Beckman Instruments, Eden Prairie, Minn.). The pelletized bacteria were washed two times with 1,000 ml physiological saline (0.85%) to remove contaminating culture media proteins.

The bacteria were resuspended in tris-buffered saline (TBS) containing 2.0% sodium N-lauroyl sarcosinate (SARKOSYL™, Sigma Chemical Co., St. Louis, Mo.), optical density 5%, 540 nm. The suspension was incubated at 4° C. for 45 minutes with continuous stirring. The cells were then disrupted using a continuous-flow cell sonicator (Banson 450, Danbury, Conn.) at 4° C., with a maximum flow rate of 5 gph. The disrupted cell suspension was centrifuged at 16,000×g for 20 minutes.

The effluent from the continuous-flow cell sonicator containing the outer membrane proteins was collected and concentrated using ethanol precipitation at −20° C. It is understood that the supernatant may also be concentrated by membrane concentration using a high capacity benchtop filtration system, such as Model DC10L with a 30,000 MW cut off spiral cartridge (Amicon, Danvers, Mass.). The concentrated material (10% T at 540 nm) was solubilized using 0.2 percent sodium dodecyl sulfate (SDS) in TBS at pH 7.4.

The elution profile of the concentrated material treated with 0.2% SDS is shown in FIG. 2. The solubilized material was applied to a Vantage column (Amicon, Danvers, Mass.) containing 3.2-L of cellufine fast flow GC-700 gel matrix (Amicon, Danvers, Mass.) equilibrated with TBS containing 0.2% SDS at 25° C. Purification of the protein was monitored by UV absorption at 280 nm. Flow rate through the column was 3,000 ml/hr and 20-ml fractions were collected using a UA-5 Detector and Retriever 5 fraction collector (ISCO, Inc., Lincoln, Neb.). Fractions from each peak were pooled and concentrated using a Diaflo ultrafiltration apparatus with a 50,000 MWCO membrane. Concentrated material from each peak was examined by gel electrophoresis. As shown in FIG. 2, peak 1 contained approximately 85% pure siderophore proteins. This solution was ethanol precipitated at −20° C. for 24 hours to remove the SDS, and then resuspended in phosphate buffered saline. The amount of protein was determined using a Pierce BCA protein assay (Pierce, Rockford, Il.).

The precipitate containing siderophore receptor proteins of *P. multocida* serotype A:3, was resuspended in physiological saline (0.85%) containing 0.1% formalin as a preservative. The protein concentration was 2500 μg/ml.

The implant was loaded with the SRPs from *P. multocida*, such that the implant had a protein concentration of 100 μg/mg or 1000 μg SRP per implant. Twenty 1-day old poults (test birds) were implanted with the 60-day release, protein-loaded implant. Forty birds received a placebo implant, and were used as controls.

FIG. 3 shows the decay pattern in the maternal antibody to SRPs from 7–28 days in both the vaccinated and non-vaccinated groups. The maternal antibody levels were measured by ELISA using SRPs as the capture molecule. At 49 days after implantation, a rise in SRP titer was observed in both groups. This increase in SRP titers was likely due to a non-specific challenge with an organism that expressed SRPs that have been shown to cross-react with the SRPs from *P. multocida*. The birds in the vaccinated group showed a significantly higher SRP titer in comparison to the non-vaccinated control birds. This increased response indicates that there was a priming effect by the release of SRP immunogen from the implant in the SRP-implanted birds that resulted in a secondary immune response from the natural field exposure with an organism expressing SRPs which acted as a booster to stimulate antibody production.

At 70 days following implantation, all birds were challenged by intramuscular injection of 1-ml of sterile saline containing 659 colony forming units (CFU) of *P. multocida* ATCC P-1059. Mortality was recorded daily for two weeks post-challenge.

Table 1, below, shows the mortality between the vaccinated and non-vaccinated turkeys following challenge with virulent *Pasteurella multocida* P-1059.

TABLE 1

Number of dead/total tested (%)	
Non-Vaccinated	SRP-Vaccinated
23/38 (61%)	5/16 (31%)

Twenty-three (61%) of the non-vaccinated birds died within 14 days after challenge showing only a 39% livability. In contrast, only 5 out of 16 birds (31%) of the SRP-vaccinated group died, with a liveability of 69%.

These results demonstrate that a vaccine presented to a bird at 1-day of age in the form of an implant matrix and the sustained release of protein immunogen from the implant in the presence of maternal antibody, effectively induced a priming response in the birds to provide protection to a later challenge by a pathogenic organism. In particular, the observed increase in antibody titers when the birds were field challenged at 49 days after implantation, shows that the release of the immunogen from the implant effectively primed the birds in the presence of maternal antibodies so that the birds were capable of producing antibodies in a secondary immune response when maternal antibody titers no longer provided effective protection against the pathogen.

An implant incorporating a siderophore receptor protein (SRP) is useful for achieving clinical efficacy of cross-reactive and cross-protective immunization against two or more different strains, species and/or genera of gram-negative bacteria or other organisms capable of expressing SRPs. An implant containing an SRP reactive with an aerobactin siderophore, an enterochelin siderophore, a citrate siderophore, a multocidin siderophore, and/or a ferrichrome siderophore, may be used to stimulate production of antibodies that cross-react with a number of different bacteria that express one or more of these receptor proteins. The effectiveness of the implant is due, at least in part, to the conservative nature of the outer membrane SRPs which are cross-reactive with siderophores produced by two or more different species, strains and/or genera of Enterobacteriaceae such as *E. coli*, Salmonella, and other gram-negative bacteria within other families such as Pasteurella and/or Pseudomonas.

EXAMPLE 3

Priming with Implant Containing Bovine Serum Albumin

Twelve 1-day old turkey poults were each administered a 21-day release, cholesterol-based, metabolizable implant (10 mg; Innovative Research, Toledo, Ohio). Six of the birds (i.e., test birds) were implanted with the implant loaded with 250 μg/mg bovine serum albumin (BSA) (Sigma). The six remaining birds received a placebo implant with no BSA, and were used as controls. Sero-conversion to BSA was monitored at 7, 21, 35, 49, 56 and 66 days post implantation and compared to the control birds. At 66 days following implantation, the birds were challenged by intravenously injecting 1-ml of a solution of BSA in physiological saline (1000 μg/ml).

As shown in FIG. 4, all BSA-implanted test birds sero-converted, and showed a significant secondary immune response after challenge as compared to the non-vaccinated controls.

EXAMPLE 4

Priming 2-week Old Turkeys with Bovine Serum Albumin (BSA)

Twenty-four 2-week old turkeys were equally divided into two groups of twelve and implanted with a cholesterol-based, metabolizable implant as described in Example 1. The first group of birds received a 60-day release implant, six of the birds (i.e., test birds) receiving the implant loaded with 500 μg BSA, and the remaining six birds (i.e., control birds) receiving a placebo implant. The second group of birds received a 90-day release implant, six of the birds (i.e., test birds) receiving the implant loaded with 1000 μg BSA, and the remaining six birds (i.e., control birds) receiving a placebo implant.

Sero-conversion of the birds to BSA was monitored at 7, 21, 35, 49, 56 and 66 days post-implantation and compared to their corresponding controls. At 66 days following implantation, the birds were challenged by intravenously injecting 1-ml of sterile saline containing 1000 μg/ml BSA.

As shown in FIGS. 5–7, all BSA-implanted test birds sero-converted as compared to the control birds, and showed a significant secondary immune response after challenge as compared to the non-vaccinated controls.

EXAMPLE 5

Priming with Vasoactive Intestinal Peptide (VIP)

A cholesterol-based implant was prepared with vasoactive intestinal peptide, and administered to breeder hens to control broodiness or nesting behavior.

Vasoactive intestinal peptide (1 mg; purified; Peninsula Laboratory Inc., Belmont, Calif.) was conjugated to 1 mg keyhole limpet hemocyanin/MPS at a ratio of (1:1), according to the method of Lerner et al., *Proc. Natl. Acad. Sci.* 78:3403–3407 (1981). Briefly, keyhole limpet hemocyanin (KLH, Calbiochem) and m-maleimidobenzoyl-N-hydroxysuccinimide (MBS, Calbiochem) were dissolved in a carrier by adding 5 mg KLH to 250 ml of 0.05M phosphate buffer (pH 6.0) and 5 mg MBS to 100 ml N,N,-dimethylformamide (Sigma). Ten μl of the MBS solution and 200 μl of the KLH solution were combined and incubated at 25° C. for 30 minutes. The KLH/MBS suspension was then loaded onto a G-25 column matrix, and fractions absorbing at 280 nm were pooled and used for conjugation.

The VIP (3.6 mg) was solubilized in dH_2O and added to 3.6 mg of the MBS/KLH suspension in 0.05M phosphate buffer (pH 7.0) at a 1:1 ratio and incubated at 25° C. for 3 hours. The resulting VIP-KLH/MBS conjugate was incorporated into 21- and 60-day release, cholesterol-based, metabolizable implants at 160 μg/capsule (Innovative Research of America Toledo, Ohio).

Twelve 15-week old breeder hens were divided into two groups of six, designated as Groups A and B. In Group A, four birds received the VIP-KLH/MBS conjugate, two birds received the 21-day implant and two birds received the 60-day release implant. The two remaining birds in Group A remained as non-implanted controls. All six birds in Group B received a placebo implant and remained as controls.

At three weeks from the expiration date of the 21-day release implant, the two 21-day implanted birds in Group A each received a 60-day release implant containing 160 μg VIP-KLH/MBS conjugate, giving these birds an additional 60 days of treatment.

Beginning at 22 weeks, the hens began to lay eggs. Eggs were collected from the hens at daily intervals for 10 weeks of production. Over that time, the VIP-treated birds produced 180 eggs. By comparison, the untreated control group produced only 16 eggs in that time period.

FIGS. 5 and 6 represent the daily and weekly egg production between the VIP-treated birds and the non-treated control group. In the VIP group, all four implanted birds went into production. By comparison, the two control birds in this group, under identical conditions, did not lay any eggs. Only two of the six birds in the control group laid eggs. It was also observed that the VIP-containing implants did not induce any granuloma formation or any other adverse reaction in the VIP-implanted birds.

EXAMPLE 6

Priming Turkeys at 1-Day of Age with Hemorrhagic Enteritis Virus (HEV)

Thirty 1-day old turkey poults were each administered a 60-day release, cholesterol-based metabolizable implant. Fifteen of the birds received the implant loaded with 10^5 $TCID_{50}$ killed HEV particles. The remaining fifteen birds received a placebo implant with no HEV and were used as controls. At seventy days after implantation, all of the birds were challenged by intravenously injecting 1-ml of sterile saline containing 10^2 $TCID_{50}$ virulent HEV particles. Mortality was recorded daily for two weeks post-challenge.

Table 2, below, shows the mortality between the vaccinated and non-vaccinated birds following an HEV challenge.

TABLE 2

Numbers of dead/total tested	
Non-Vaccinated	Vaccinated
6/15 (40%)	0/15

Six (40%) of the non-vaccinated birds died within 14 days after challenge. By comparison, none of the birds in the vaccinated group died. These results demonstrate that a vaccine presented to a bird at 1-day of age in the form of a

metabolizable implant can induce effective protection to a later viral challenge.

What is claimed is:

1. A method of inducing an immune response in a 1-day old fowl, comprising:

administering to the fowl, a biocompatible solid implant that is bioabsorbable, biodegradable, bioerodible, or a combination thereof, with an immunogen releasably contained therein by subcutaneous or intermuscular injection;

wherein the immunogen is derived from a pathogenic organism selected from the group consisting of viruses, bacteria, fungi, molds, protozoans, nematodes, helminthes, and spirochetes;

the implant providing sustained release of the immunogen into tissue fluids of the fowl for a period of about 1–90 days, in an amount effective to stimulate a primary immune response to the immunogen in the fowl in the presence of circulating maternal antibodies;

the primary immune response effective to stimulate a secondary immune response in the fowl upon subsequent contact with the immunogen.

2. The method according to claim 1, wherein the secondary immune response provides an about 5–100 fold increase in anti-immunogen antibody titers in the animal.

3. The method according to claim 2, wherein said increase in antibody titers is at about 6–48 hours after the contact with the immunogen.

4. The method according to claim 1, wherein the animal is administered an implant containing about 25–5000 μg of the immunogen.

5. The method according to claim 1, wherein the implant provides a release rate of the immunogen of about 1–100 μg per day.

6. The method according to claim 1, wherein the animal is administered an implant comprising a polymer that is bioabsorbable, biodegradable, bioerodible, or combination thereof.

7. The method according to claim 6, wherein the polymer is selected from the group consisting of a cellulosic polymer, polylactic acid, polyglycolic acid, polycaprolactone, polyanhydride, polyamide, and copolymers thereof.

8. The method according to claim 1, wherein the animal is administered a cholesterol-based implant.

9. The method according to claim 1, wherein the animal is administered an implant comprising a non-erodible synthetic polymer selected from the group consisting of a polyethylene, and ethylene-acetate copolymer.

10. The method according to claim 1, wherein the immunogen is selected from the group consisting of peptides, polypeptides, proteins, glycoproteins, polysaccharides, lipopolysaccharides, sphingolipids, toxins, and anti-idiotype antibodies.

11. The method according to claim 1, wherein the immunogen is derived from a virus or bacterium.

12. The method according to claim 11, wherein the immunogen is derived from a virus selected from the group consisting of New Castle disease virus, hemorrhagic enteriditis virus, infectious rhinotracheitis virus, infectious bursal disease virus, infectious bronchitis virus, avian encephalomyelitis virus, bovine viral diarrhea virus, bovine respiratory syncytial virus, bog cholera virus, equine encephalomyelitis virus, canine distemper virus, fowl pox virus, rabies virus, avian leukosis virus, and avian influenza virus.

13. The method according to claim 11, wherein the immunogen is derived from a bacterium selected from the group consisting of *Escherichia coli*, Salmonella, Pasteurella, Pseudomonas, Klebsiella, Actinobacillus, Haemophilus, Streptococcus, Bordetella, Staphylococcus, Clostridia, Erysipelothrix, and Borrelia.

14. The method according to claim 1, wherein the immunogen is derived from a fungi or mold selected from the group consisting of Aspergillus, Penicillium, Fusarium, Rhizopus, *Aspergillus fumigatis*, and Candida.

15. The method according to claim 10, wherein the immunogen is a polypeptide capable of functioning in the transport of iron across a cell membrane of an organism.

16. The method according to claim 15, wherein the polypeptide is a siderophore receptor protein reactive with a siderophore selected from the group consisting of aerobactin, enterochelin, citrate, multocidin, ferrichrome, coprogen, and mycobactin.

17. The method according to claim 1, wherein the immunogen is vasoactive intestinal peptide.

18. The method according to claim 1, wherein the fowl is a turkey or chicken.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
Certificate

Patent No. 5,538,733 Patented: July 23, 1996

On petition requesting issuance of a certificate for correction of inventorship pursuant to 35 U.S.C. 256, it has been found that the above identified patent, through error and without any deceptive intent, improperly sets forth the inventorship.

Accordingly, it is hereby certified that the correct inventorship of this patent is: Daryll A. Emery, Willmar, MN (US); and Darren E. Straub, Willmar, MN (US).

Signed and Sealed this Sixteenth Day of January 2007.

WILLIAM R. DIXON, JR.
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Technology Center 1600

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Emery et al.

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[54] **ACTIVE IMMUNIZATION USING A SIDEROPHORE RECEPTOR PROTEIN**

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[73] Assignee: **Willmar Poultry Company, Inc.**, Willmar, Minn.

[21] Appl. No.: 385,273

[22] Filed: Feb. 8, 1995

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 194,040, Feb. 9, 1994, abandoned.

[51] **Int. Cl.**[6] A61K 39/102; A61K 39/02; A61K 39/116; A61K 39/108

[52] **U.S. Cl.** 424/255.1; 424/184.1; 424/203.1; 424/282.1; 424/93.3; 424/826; 424/823; 424/824; 424/241.1

[58] **Field of Search** 424/203.1, 255.1, 424/257.1, 241.1, 258.1, 282.1, 93.3

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Primary Examiner—Hazel F. Sidberry
Attorney, Agent, or Firm—Merchant, Gould, Smith, Edell, Welter & Schmidt, P.A.

[57] **ABSTRACT**

The invention provides a vaccine for immunizing poultry and other animals against infection by a gram-negative bacteria, and a method of immunizing an animal using the vaccine. The vaccine may contain purified siderophore receptor proteins derived from a single strain or species of gram-negative bacteria or other organism, which are cross-reactive with siderophores produced by two or more strains, species or genera of gram-negative bacteria. The invention further provides a process for isolating and purifying the siderophore receptor proteins, and for preparing a vaccine containing the proteins. Also provided is a method for diagnosing gram-negative sepsis.

16 Claims, 9 Drawing Sheets

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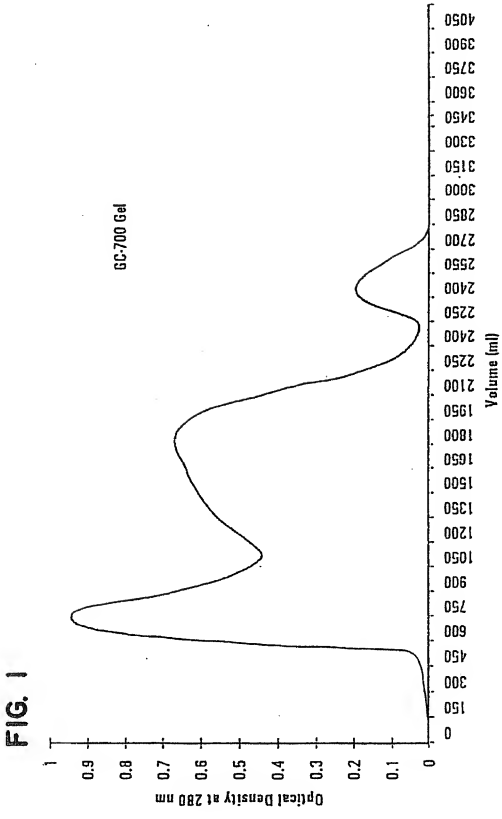

GC-700 Gel
Optical Density at 280 nm
Volume (ml)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
150
300
450
600
750
900
1050
1200
1350
1500
1650
1800
1950
2100
2250
2400
2550
2700
2850
3000
3150
3300
3450
3600
3750
3900
4050

FIG. 1

FIG. 2

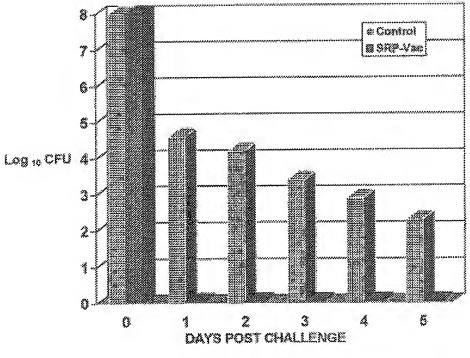

Control
SRP-Vac
Log 10 CFU
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
DAYS POST CHALLENGE

FIG. 3

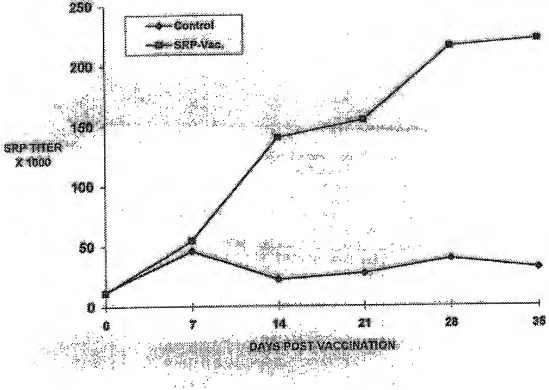

Control
SRP-Vac.
250
200
150
100
50
0
SRP TITER X 1000
0
7
14
21
28
35
DAYS POST VACCINATION

FIG. 4

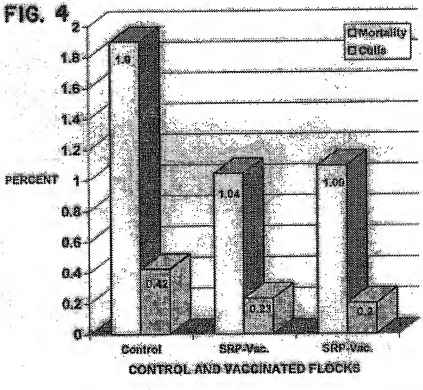

Mortality
Culls
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PERCENT
1.8
0.42
1.04
0.23
1.00
0.2
Control
SRP-Vac.
SRP-Vac.
CONTROL AND VACCINATED FLOCKS

FIG. 5

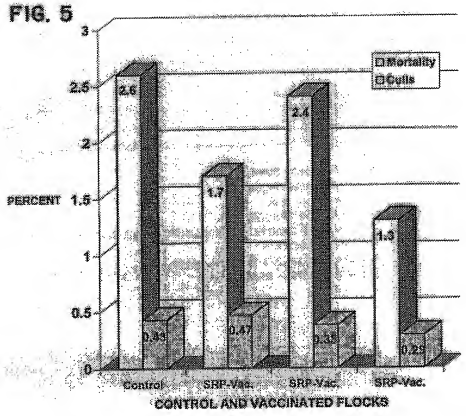

Mortality
Cells
PERCENT
3
2.5
2
1.5
1
0.5
0
2.6
0.33
1.7
0.47
2.4
0.35
1.5
0.25
Control
SRP-Vac.
SRP-Vac.
SRP-Vac.
CONTROL AND VACCINATED FLOCKS

FIG. 6

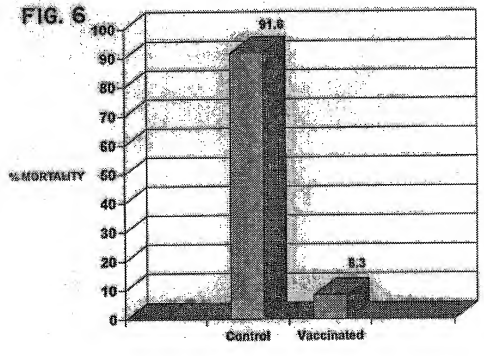

% MORTALITY
100
90
80
70
60
50
40
30
20
10
0
91.6
8.3
Control
Vaccinated

FIG. 7

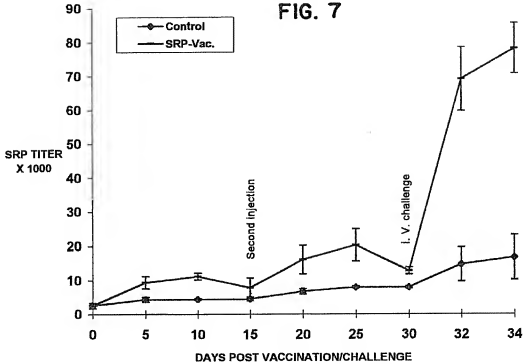

Control
SRP-Vac.
SRP TITER X 1000
Second injection
I. V. challenge
DAYS POST VACCINATION/CHALLENGE
0
10
20
30
40
50
60
70
80
90
0
5
10
15
20
25
30
32
34

FIG. 8

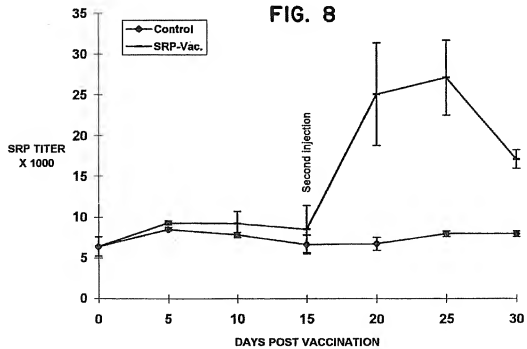

Control
SRP-Vac.
SRP TITER X 1000
Second injection
DAYS POST VACCINATION
0
5
10
15
20
25
30
35
0
5
10
15
20
25
30

FIG. 9

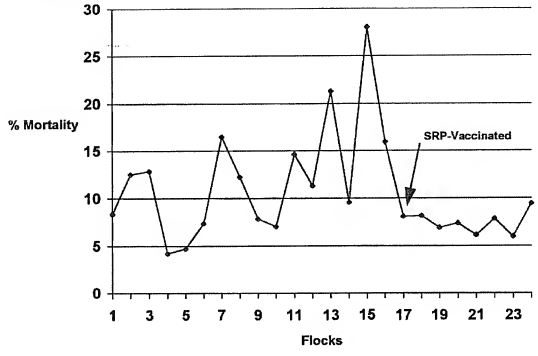

30
25
20
15
10
5
0
% Mortality
SRP-Vaccinated
1 3 5 7 9 11 13 15 17 19 21 23
Flocks

FIG. 10

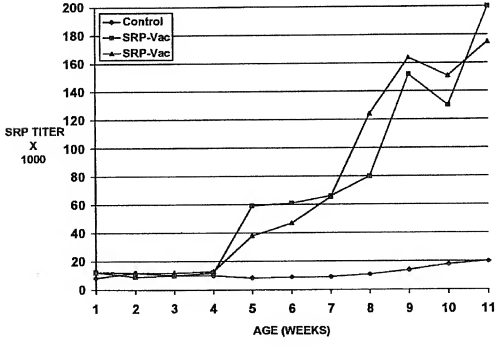

Control
SRP-Vac
SRP-Vac
200
180
160
140
120
100
80
60
40
20
0
SRP TITER
X
1000
1 2 3 4 5 6 7 8 9 10 11
AGE (WEEKS)

FIG. 11

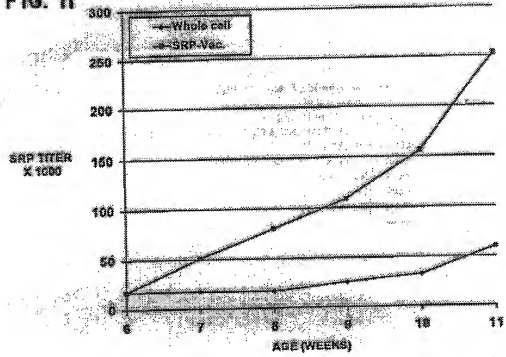

Whole cell
SRP-Vac.
SRP TITER X 1000
300
250
200
150
100
50
0
6
7
8
9
10
11
AGE (WEEKS)

FIG. 12

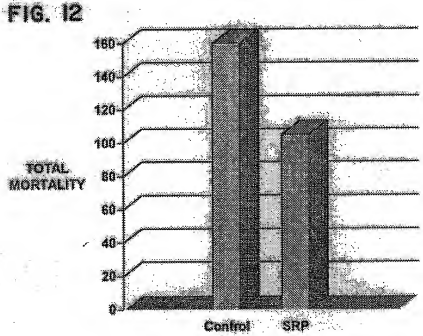

TOTAL MORTALITY
160
140
120
100
80
60
40
20
0
Control
SRP

FIG. 13

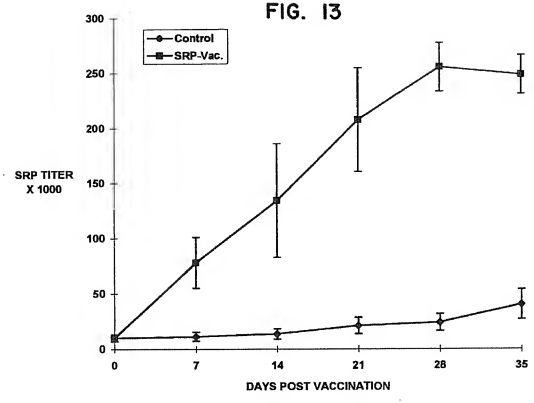

Control
SRP-Vac.
SRP TITER X 1000
300
250
200
150
100
50
0
0
7
14
21
28
35
DAYS POST VACCINATION

FIG. 14

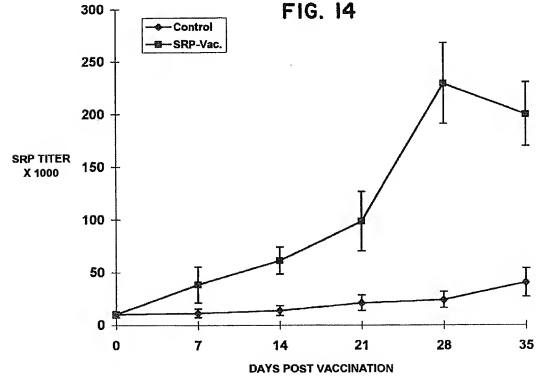

Control
SRP-Vac.
SRP TITER X 1000
300
250
200
150
100
50
0
0
7
14
21
28
35
DAYS POST VACCINATION

FIG. 15

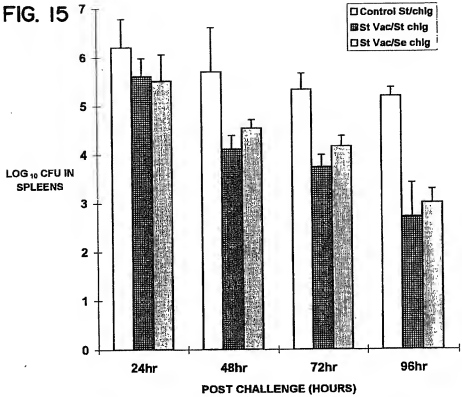
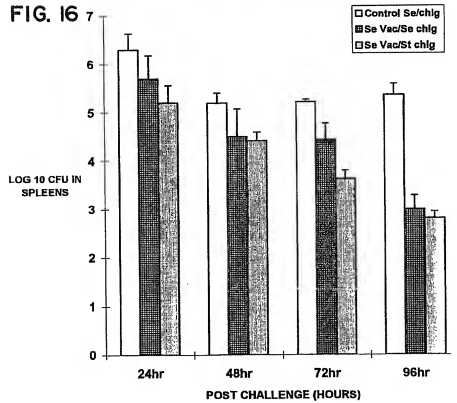

Control St/chlg
St Vac/St chlg
St Vac/Se chlg
LOG 10 CFU IN SPLEENS
0
1
2
3
4
5
6
7
24hr
48hr
72hr
96hr
POST CHALLENGE (HOURS)

FIG. 16


Control Se/chlg
Se Vac/Se chlg
Se Vac/St chlg
LOG 10 CFU IN SPLEENS
0
1
2
3
4
5
6
7
24hr
48hr
72hr
96hr
POST CHALLENGE (HOURS)

ACTIVE IMMUNIZATION USING A SIDEROPHORE RECEPTOR PROTEIN

CROSS REFERENCE TO RELATED APPLICATIONS

This patent application is a continuation-in-part of U.S. patent application Ser. No. 08/194,040, filed Feb. 9, 1994 now abandoned.

BACKGROUND OF INVENTION

The economic impact of infectious diseases in the poultry industry is well-appreciated. Immunization of birds has helped reduce the cost of production by decreasing the incidence of gastrointestinal, respiratory and systemic diseases. While vaccines provide adequate immunity for those pathogens against which a flock has been immunized, there are few vaccines which can provide broad-based cross-protection against unanticipated diseases or against those diseases for which an animal has not been specifically vaccinated.

A number of important diseases of domestic poultry are caused by bacteria able to invade host tissues, such as *Salmonella spp., Escherichia spp.* and *Pasteurella spp.* While many vaccines are available for immunization against individual species and serotypes, none provide cross-protection or stimulate broad-based immunity against multiple serotypes, species or genera.

One essential factor required for a bacteria to induce clinical disease is the ability to proliferate successfully in a host tissue. Iron is an essential nutrient for the growth of gram-negative bacteria in vivo, but is virtually unavailable in mammalian and/or avian tissues because the iron is either intracellular or extracellular, complexed with high affinity, iron-binding proteins, for example, transferring in blood and lymph fluids and lactoferrin in external secretions. In normal tissues, the concentration of iron is approximately 10^{-18}M, far below that required for bacterial growth.

To circumvent these restrictive conditions, pathogenic bacteria have evolved high affinity iron transport systems produced under low iron conditions, which consist of specific ferric iron chelaters, "siderophores," and iron-regulated outer membrane proteins (IROMPs) and/or siderophore receptor proteins (SRPs) which are receptors for siderophores on the outer membrane of the bacterial cell. Siderophores are synthesized by and secreted from the cells of gram-negative bacteria under conditions of low iron. Siderophores are low molecular weight proteins ranging in molecular mass from about 500 to about 1000 MW, which chelate ferric iron and then bind to IROMPs in the outer bacterial membrane which, in turn, transport the iron into the bacterial cell. Although the use of IROMPs as immunogens has been considered, these proteins have not been examined for such use, at least in part, due to an inability to extract these proteins from bacterial membranes in high volume and with a desired level of purity and immunogenic quality.

Accordingly, an object of the invention is to provide a method for obtaining high amounts of immunogenic quality siderophore receptor proteins from *Escherichia coli*, Salmonella, Pasteurella, and other gram-negative bacteria. Another object is to provide a vaccine for immunizing poultry and other animals against these bacteria. Yet another object is to provide a vaccine for cross-protection against multiple serotypes, species and/or genera of bacteria belonging to the family Enterobacteriaceae and/or Pasteurellaceae. A further object is to provide a diagnostic assay to monitor and/or profile sepsis and subclinical disease caused by gram-negative bacteria under field conditions.

SUMMARY OF THE INVENTION

These and other objects are achieved by the present invention which is directed to a vaccine for prevention and treatment of infection by gram-negative bacteria, and a method of immunizing poultry and other animals against such infections using the vaccine. The invention also provides a method for isolating and purifying outer membrane siderophore receptor proteins from gram-negative bacteria for producing the vaccine. The invention further provides an in vitro method of diagnosing infections of gram-negative bacteria in an animal using antibodies raised to the isolated receptor proteins.

The vaccine is useful for immunizing an avian or other animal against infection by gram-negative bacteria such as colibacillosis, salmonellosis and pasteurellosis. The vaccine is composed of a substantially pure siderophore receptor protein derived from the outer membrane of a gram-negative bacteria, for example, *Salmonella spp., Escherichia spp.* and *Pasteurella spp.* A siderophore receptor protein, useful according to the invention, is a protein or antigenic peptide sequence thereof derived from the outer membrane of a gram negative bacterium, which is capable of producing an antibody that will react with the siderophore receptor protein expressed by a gram-negative bacteria of the same or different strain, species or genus. Preferably, the siderophore receptor protein is derived from a bacterium belonging to the family Enterobacteriaceae and/or Pasteurellaceae.

The vaccine contains siderophore receptor proteins (SRPs) derived from a gram-negative bacteria, capable of eliciting an immune response in an animal with the production of anti-SRP antibodies. These antibodies will react with siderophore receptor proteins of that bacteria, and may also cross-react with siderophore receptor proteins of a different strain, species and/or genera of gram-negative bacteria to provide cross-protection against infection from such other bacteria. Useful siderophore receptor proteins having a molecular weight of about 72–96 kDa, as determined by SDS-PAGE, have been isolated from *E. coli, Salmonella spp., Pasteurella spp., Pseudomonas spp.*, and *Klebsiella spp.* Preferably, the siderophore receptor proteins (SRPS) are derived from *Escherichia coli, Salmonella spp.* and/or *Pasteurella spp.* The antibodies produced from those SRPs will react with SRPs of those bacteria and cross-react with SRPs of a different strain, species and/or genera of bacteria within the family Enterobacteriaceae and/or Pasteurellaceae.

The vaccine contains one or more siderophore receptor proteins extracted from the outer membrane of a single strain or species, or two or more different strains or species of gram-negative bacteria. The amount and type of siderophore receptor protein included in the vaccine is effective to stimulate production of antibodies reactive with a siderophore receptor protein of one, preferably two or more strains, species or genera of gram-negative bacteria. A preferred vaccine is composed of an amount and profile of siderophore receptor proteins to effectively induce antibodies reactive with a majority, preferably all, of the siderophore receptor proteins of a bacterial population to effectively enhance opsonization and complement-mediated bacterial lysis, and/or block the iron binding capacity of the bacteria. The siderophore receptor protein is combined with a physiologically-acceptable carrier, preferably a liquid. The vaccine may further include an adjuvant to enhance the immune response, and other additives as desired, such as preservatives, flavoring agents, buffering agents, and the like.

The present invention also provides a method for isolating high quantities of immunogenically effective siderophore

receptor proteins from outer membranes of a single strain or species of gram-negative bacteria such as *E. coli*, Salmonella and/or Pasteurella. The method includes culturing the organism under conditions of low iron availability, that is, in a culture medium that lacks iron or includes an iron chelating agent. The siderophore receptor proteins are then separated from the bacterial outer membrane and purified by use of the anionic detergent, sodium dodecyl sulfate, preferably under non-reducing conditions.

The siderophore receptor proteins may be utilized to raise polyclonal antibody sera and monoclonal antibodies for use in passive immunization therapies. Such antibodies may also be used in an in vitro method of diagnosing a gram-negative bacterial infection in an animal. The diagnostic method includes contacting a body material potentially infected with a gram-negative bacteria, such as a tissue sample or body fluid, with a labelled antibody raised to a siderophore receptor protein, and detecting the label in the complex formed between the siderophore receptor protein in the body material and the labelled antibody. The method may also be performed by combining the body sample with the antibody to the siderophore receptor protein, and then contacting the sample with a labelled anti-species antibody reactive with the protein-specific antibody, and then detecting the label.

The siderophore receptor proteins car also be used as capture antigens in a method of monitoring and profiling gram negative sepsis. For example, the protein may be used in an ELISA technique in which the protein is bound to a solid support and contacted with a sample material to react with and detect antibodies present in the sample.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a graphic depiction of the elution profile of concentrated, solubilized siderophore receptor proteins isolated from *Escherichia coli* serotype 078 (ATCC 55652).

FIG. **2** is a graphic depiction of the quantitative clearance of *Salmonella agona* in spleens of turkeys vaccinated with IROMPs isolated from *E. coli* and non-vaccinated controls.

FIG. **3** is a graphic depiction of the serological response to *E. coli* siderophore receptor proteins (SRPS) between vaccinated and non-vaccinated flocks.

FIG. **4** is a depiction of the total % mortality and culls in control and *E. coli* SRP-vaccinated flocks (3–13 weeks of age).

FIG. **5** is a depiction of the total % mortality and culls in control and *E. coli* SRP-vaccinated flocks (3–13 weeks of age).

FIG. **6** is a graphic depiction of the total mortality in SRP-vaccinated and non-vaccinated turkeys following challenge with *Pasteurella multocida* P-1059.

FIG. **7** is a graphic depiction of the serological response in hirds vaccinated with purified siderophore receptor proteins from *Salmonella senftenberg*, showing cross-reactivity with the SRP of *E. coli.*

FIG **8** is a graphical depiction of the serological response in birds vaccinated with purified siderophore receptor proteins from *P. Multocida*, showing cross-reactivity with the SRP of *E. coli.*

FIG. **9** is a graphic depiction of the total % mortality in consecutive flocks before and after vaccinating with siderophore receptor proteins derived from *E. coli* 078.

FIG. **10** is a graphic depiction of the serological response to SRPs from *E. coli* between SRP-vaccinated and non-SRP-vaccinated commercial turkey flocks.

FIG. **11** is a graphical depiction of the serological response of purified SRP and whole cell of *Salmonella heidelberg.*

FIG. **12** is a graphic depiction of the total mortality between progeny of SRP-vaccinated and non-vaccinated (control) breeder hens.

FIG. **13** is a graphical depiction of the serological response in hirds vaccinated with purified siderophore receptor proteins from *Salmonella typhimurium*, showing cross-reactivity with the SRP of *E. coli.*

FIG. **14** is a graphical depiction of the serological response in hirds vaccinated with purified siderophore receptor proteins from *Salmonella enteritidis*, showing cross-reactivity with the SRP of *E. coli.*

FIG. **15** is a graphical depiction of SRPs of *Salmonella typhimurium* as a protective immunogen against a homologous and heterologous challenge in turkeys.

FIG. **16** is a graphical depiction of SRPs of *Salmonella enteritidis* as protective immunogens against a homologous and heterologous challenge in turkeys.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "substantially pure" means that the siderophore receptor protein has been extracted and isolated from its natural association with other proteins, lipids, and other like substances and elements of a bacterial cell or other organism.

Gram-negative hacteria are frequent pathogens of poultry and other animals, such as domestic foul, livestock, horses, companion animals, and humans. In an iron-restricted environment, bacteria such as *Escherichia coli, Salmonella spp.* and *Pasteurella spp.* produce siderophores that chelate ferric iron and bind to outer membrane proteins that function as siderophore receptors on the bacterial membrane.

The inveation provides an improved process for isolating and separating siderophore receptor proteins from the outer membrane of gram-negative bacteria. Isolation and purification of immunogenically intact siderophore receptor proteins from bacterial membranes in a sufficient quantity and immunogenic quality for formulating a vaccine against infection by gram-negative bacteria has been difficult. The structural orientation, or conformation, of the outer membrane protein necessary to provide antigenicity may be typically lost when the protein is separated and purified from the lipopolysaccharide complex. Another problem is that the protein becomes denatured by the separation process wherein its immunogenicity is lost. According to the present invention, however, the isolation and separation of immunogenic quantites of antigenically effective siderophore receptor proteins from the outer membrane of gram-negative bacteria has been achieved. This enables the production of vaccines and hyperimmunized sera for the treatment of animals infected or susceptible to infection by gram-negative bacteria, and in vitro diagnostic methods for detecting such an infection in an animal.

As a group, gram-negative hacteria possess a common cell wall structure. Components of the cell wall structure may be used as immunogens. However, these immunogens may provide only homologous immune protection. The present vaccine utilizes a combination of outer membrane siderophore receptor proteins common to two or more gram-negative hacteria that are capable of proliferating in the blood or host tissues and causing infection in an animal. The vaccine may contain two or more siderophore receptor proteins (SRPs), preferably four or more SRPs derived from the outer membrane of one or more strains or species of gram-negative hacteria and/or other organism. Preferably, the SRPs are derived from a single strain or species of

gram-negative bacteria. A preferred siderophore receptor protein for use in the vaccine has a common receptor reactive with siderophores produced by two or more strains, species and/or genera of gram-negative bacteria.

An example of a useful siderophore receptor protein is the receptor protein for aerobactin (MW about 72–74 kDa) produced by members of the family Enterobacteriaceae, for example, *Escherichia coli*, Salmonella and Klebsiella. Antibodies produced against an aerobactin receptor protein of one species, strain or genus of that family have been found to cross-react with other bacteria within the family. Species of Pseudomonas of the family Pseudomonadaceae also express aerobactin siderophore receptor proteins that can be isolated according to the invention and used in a vaccine to produce antibodies that cross-react with the aerobactin receptor proteins of *E. coli*, Salmonella and Klebsiella, among other members of the family Enterobacteriaceae.

Another example of a suitable siderophore receptor protein for use in the present vaccines is that produced by *Pasteurella multocida* for the siderophore multocidin (MW about 500–1000 kDa). Antibodies to the multocidin receptor protein will react with all three of the SRPs in *Pasteurella multocida*. In Western blots, two of the larger siderophore proteins (96 kDa, 84 kDa) of *P. multocida* showed reactivity with hyperimmune *E. coli* protein antisera. Antibodies produced to multocidin receptor proteins will cross-react with the siderophore receptor proteins of *Salmonella spp.* and *E. coli*, as demonstrated by ELISA and Western blot analysis.

Other siderophore receptor proteins include those reactive with the siderophore enterochelin (MW about 81–84 kDa) produced by *E. coli*, Salmonella, Pseudomonas and Klebsiella; and the siderophore citrate (MW about 74–78 kDa) produced by *E. coli*, among others. A vaccine containing the enterochelin and/or citrate receptor proteins will produce antibodies reactive with *E. coli*, Salmonella and other bacteria of the family Enterobacteriaceae, and with Pseudomonas of the family Pseudomonadaceae.

Another useful SRP is the siderophore receptor protein for ferrichrome (MW about 78 kDa) produced by *E. coli*, and *Salmonella spp.* In commercial poultry raising facilities, infection by Aspergillus causes serious respiratory problems in the birds. In the lungs, Aspergillus will excrete ferrichrome to acquire iron as a growth nutrient. Under iron restriction or systemic conditions, *E. coli* and Salmonella will express ferrichrome receptor protein. They are also opportunistic bacteria that can scavenge and utilize ferrichrome produced by Aspergillus as a growth nutrient. Therefore, it is preferred that the vaccine preparation include a ferrichrome receptor protein to induce antibodies that will bind and cross-react with the ferrichrome receptor proteins of gram-negative bacteria including *E. coli* and Salmonella, and fungi/mold. A vaccine containing this SRP will elicit an immune response to the protein to enhance the bactericidal activity of the antibody. Also, once the avian or other animal is vaccinated with a ferrichrome receptor protein, Aspergillus expressing this protein in vivo in the animal will enhance the antibody response to the ferrichrome receptor protein which in turn will cross-react with Salmonella and *E. coli* and other bacteria that express the ferrichrome receptor protein.

Antibody elicited from a ferrichrome receptor protein (MW about 78 kDa) derived from *E. coli* can cross-react with the receptor proteins of fungi, such as *Aspergillus flavus, Aspergillus fumigatus*, Penicillium and Fusarium. Western blot analysis against the outer membrane proteins (OMPs) of *A. fumigatus* using anti-SRP antibody revealed three cross-reactive proteins (MW about 45–90 kDa). The inclusion of a ferrichrome receptor protein into a vaccine preparation will provide inducement of antibodies that will react with the fungi and/or bacteria to prevent binding and excretion of the ferrichrome siderophore. Animals such as birds that are vaccinated with a vaccine preparation containing a ferrichrome receptor protein will get an elevated antibody titer by bacteria and/or fungi that challenge the animal and produce a ferrichrome receptor protein. Also, antibody to the ferrichrome receptor can be elevated by natural field challenge by bacteria or fungi which can induce a bactericidal effect that could lessen system challenge and disease potential.

Yet another useful SRP is a coprogen receptor protein (MW about 74–76 kDa) produced by *E. coli*. Antibodies produced against coprogen receptor protein will cross-react with the SRPs of other *E. coli* expressing this protein under systemic conditions.

In one embodiment, the vaccine is formulated with siderophore receptor proteins (SRPs) of different types and/or molecular weights, derived from a first gram-negative bacteria, the SRPs being capable of stimulating production of antibodies that react with the first gram-negative bacteria as well as a second gram-negative bacteria of a different strain or species than the first gram-negative bacteria. The vaccine preferably contains all SRPs derived from the gram-negative bacteria infectious agent. For example, *P. multocida* and *Salmonella spp.* have been identified as producing 3 SRPs each, and *E. coli* produced 2, 3, 4, and 6 SRPs varying between serotypes. Accordingly, the vaccine is formulated to contain the SRPs derived from the bacterial causative agent, i.e., 2–6 or more SRPs. It is preferred that the vaccine also include siderophore receptor proteins of different types and/or molecular weights derived from a gram-negative bacteria of a strain or species different than the first gram-negative bacteria, preferably 1–15 SRPs, preferably 5–10 SRPs.

For example, the vaccine may contain a siderophore receptor protein derived from *E. coli*, preferably *E. coli* serotype 01a, 02a and/or 078, that is capable of stimulating production of an antibody immunoreactive with that *E. coli* and a second gram-negative bacteria such as *Salmonella spp., Pseudomonas aeruginosa, Klebsiella pneumoniae* and/or *Pasteurella multocida*. In another example, the vaccine may contain a siderophore receptor protein derived from a species of Pasteurella, such as *P. multocida*, that is capable of stimulating production of an antibody immunoreactive with that species of Pasteurella and a second gram-negative bacteria such as *Salmonella spp.* and/or *E. coli*. In yet another example, the vaccine may contain a siderophore receptor protein derived from a species of Salmonella that is capable of stimulating production of an antibody immunoreactive with that species of that species of Salmonella, and a second gram-negative bacteria such as *E. coli*, Pseudomonas, Klebsiella, and/or *Pasteurella multocida*.

A vaccine formulated with siderophore receptor proteins derived from *E. coli* is preferably composed of an aerobactin, ferrichrome, coprogen, enterochelin and/or citrate SRP, having molecular weights of about 89 kDa to about 72 kDa, as determined by SDS-PAGE. The vaccine preferably includes 2–5 receptor proteins, preferably 3–5 proteins, preferably all five *E. coli* SRPs. A preferred vaccine against *E. coli* infection is prepared with the SRPs from *E. coli* 078 (ATCC #55652). *E. coli* 078 has been identified as producing up to 6 SRPs ranging in molecular weight from about 72 to 90 to 92 kDa, as determined by SDS-PAGE. The SRPs derived from *E. coli* 078 include aerobactin, ferrichrome,

coprogen, enterochelin and citrate SRPs, having molecular weights of about 91–92 kDa, 89 kDa, 84 kDa, 78 kDa, 74 kDa and 72 kDa, as determined by SDS-PAGE, 12.5% acrylamide reducing gel. Although the 91–92 kDa proteins of *E. coli* 078 are expressed in culture media made with and without iron, the expression of those proteins is enhanced in an iron-restricted medium, and as used herein, the 91–92 kDa proteins are considered to be iron-regulated SRPs. A preferred vaccine for immunizing an animal against *E. coli* is formulated with an aerobactin, ferrichrome, coprogen, enterochelin and citrate SRP derived from *E. coli*, preferably *E. coli* 078, made of at least 5 siderophore receptor proteins, preferably at least 6 receptor proteins, or more, to induce anti-SRP antibodies to effectively block a majority, preferably all, iron binding sites of *E. coli* serotypes present in an infection, and to induce high antibody levels to promote bactericidal activity.

It is further preferred that the vaccine includes one or more SRPs, preferably about 1–15 SRPs, derived from one or more additional bacteria, different from the first gram-negative bacteria. For example, in a vaccine composed of SRPs from *E. coli*, it is desirable to include one or more of the SRPs derived from Salmonella, *Pasteurella multocida*, Klebsiella and/or Pseudomonas.

A preferred vaccine contains each of the SRPs of different types and/or molecular weights, of a population of gram-negative bacteria to induce production of antibodies that will effectively block the iron-binding sites of all of the various SRPs of the bacterial population so that the bacteria cannot effectively bind iron as a nutrient for growth. It is also preferred that the vaccine will induce high SRP antibody levels that will enhance opsonization and/or complement-mediated bacterial lysis. Due to the variation in iron-regulated outer membrane proteins (IROMPs) produced between and within bacterial serotypes, formulating a vaccine with SRPs isolated and purified from a single isolate source may provide only a partial profile of the SRPs present in a bacterial population. Consequently, the effectiveness of the vaccine to induce anti-SRP antibodies to block bacterial iron-binding sites and inhibit bacterial infection may be limited to those serotypes that produce all or less than all of the SRPs included in the vaccine, while those bacterial serotypes producing other SRPs may retain an iron-binding capacity. Thus, it is preferred that a profile, or handing pattern (i.e., SDS-PAGE protein separations), of a bacterial population is conducted by examining different field isolates, preferably about 25–100 isolates, to determine the SRPs that are present, and all of the various SRPs are included in the vaccine.

Non-iron regulated proteins and polypeptides may also be included in the vaccine as adjuvants to enhance the effectiveness of the vaccine and increase opsonization, that is, increase macrophage activity resulting in increased phagocytosis of antibody-bound cells, and induce complement-mediated bacterial lysis. A useful adjuvant protein is a 34–38 kDa group of outer membrane proteins (porins, i.e., pore-forming proteins) derived from gram-negative bacteria of the family Enterobacteriaceae and Pasteurellaceae including *E. coli* 078, and other gram-negative bacteria. The trans-membrane and porin proteins (MW 34–38 kDa) identified as OmpA, OmpC, OmpD and OmpF are expressed with and without iron, are relatively conserved between gram-negative bacteria, and play a role in iron binding. For example, OmpF and OmpC will bind lactoferrin (Erdei et al., *Infection and Immunity* 62:1236–1240 (April 1994)), while OmpA will bind ferrichrome (Coulion et al., *J. Gen. Microbiol.* 110:211–220 (1979)). Antibodies early in infection particularly of the IgM class will cross-react with outer membrane proteins of *E. coli*, Salmonella, Pasteurella, Pseudomonas and Klebsiella, and will bind lactoferrin and/or ferrichrome, precluding the availability of an iron source for bacterial growth. Antibodies to these proteins will also bind to the porin Omp on the surface to enhance opsonization and/or complement-mediated bacterial lysis. Immunogenically intact 34–38 kDa porin outer membrane proteins can be isolated and purified according to the process of the invention.

The vaccine may be used to immunize poultry and other animals such as domestic fowl, livestock, horses, companion animals, and humans, against infection caused by one or more gram-negative bacteria. The vaccine is effective for eliciting antibodies that are immunoreactive with a gram-negative bacteria that expresses one or more siderophore receptor protein(s).

Preferably, the vaccine is capable of achieving clinical efficacy of cross-reactive and cross-protective immunization against two or more different strains, species and/or genera of gram-negative bacteria or other organisms capable of expressing siderophore receptor proteins. For example, a vaccine containing siderophore receptor proteins for aerobactin, enterochelin, ferrichrome, coprogen and/or citrate, may be used to stimulate production of antibodies that cross-react with a number of different bacteria that express one or more of these receptor proteins. The effectiveness of the present vaccine is due, at least in part, to the conservative nature of the outer membrane siderophore receptor proteins which are cross-reactive with siderophore receptor proteins produced by two or more different species, strains and/or genera of Enterobacteriaceae such as *E. coli*, Salmonella, and other gram-negative bacteria within other families such as Pasteurella and/or Pseudomonas.

Because of the cross-reactivity of the SRPs, the vaccine is effective in stimulating production of antibodies that react with the first gram-negative bacteria (from which the SRPs were derived), as well as a second gram-negative bacteria of a different strain or species than the first gram-negative bacteria. For example, a vaccine can be formulated to contain a siderophore receptor protein derived from *E. coli*, preferably *E. coli* serotype 01a, 02a and/or 078, more preferably *E. coli* 078, that is effective in stimulating production in vivo of an antibody immunoreactive with that *E. coli* serotype (from which the SRP(s) were derived), and a second gram-negative bacteria such as *Salmonella spp., Pseudomonas aeruginosa, Klebsiella pneumoniae* and/or *Pasteurella multocida*. In another example, the vaccine can contain a siderophore receptor protein derived from a species of Pasteurella, such as *P. multocida*, that is effective in stimulating production of an antibody immunoreactive with that species of Pasteurella and a second gram-negative bacteria such as *Salmonella spp.* and/or *E. coli*. In yet another example, the vaccine can contain a siderophore receptor protein derived from a species of Salmonella that is effective in stimulating production of an antibody immunoreactive with that species of Salmonella, and a second gram-negative bacteria such as *E. coli*, Pseudomonas, Klebsiella, and/or *Pasteurella multocida*.

Advantageously, immunization using the present vaccine containing an immunogen cross-reactive with multiple species, strains and genera of gram-negative bacteria, not only minimizes immunization costs since separate inoculations with a different immunogen for each type of gram-negative bacteria is not required. In addition, the present vaccine provides protection against new strains or unanticipated pathogens of gram-negative bacteria which produce

siderophore receptor proteins that will cross react with antibodies induced by the siderophore receptor proteins contained in the vaccine. The vaccine given to an adult animal is highly efficacious in treating and preventing gram-negative sepsis not only in the adult animal but also their progeny by the direct transfer of anti-SRP antibodies.

Commercial bacterial whole cell vaccines are useful for treating a particular disease and/or infection but do not provide effective cross-protection against other infection. For example, avian pasteurellosis in turkeys caused by *Pasteurella multocida* is clinically diagnosed by particular lesions induced by the bacterial infection. Treating the disease with a commercial whole cell vaccine stimulates antibodies that are homologous but not heterologous in their action, and will not cross-protect against infection by other bacteria.

Advantageously, the present vaccines provide cross-protection against a number of infections caused by gram-negative bacteria. According to the invention, an animal species suffering from gram-negative bacterial sepsis can be administered the vaccine containing SRPs derived from the (causative agent) gram-negative bacteria to induce antibodies immunoreactive with those SRP(s) to inhibit the disease state. The antibodies will also cross-react with SRP(s) produced by another gram-negative bacteria to inhibit a disease state caused by that other bacteria. Thus, a vaccine containing SRPs of a first gram-negative bacteria will provide protection against an infection caused by that bacteria and provide cross-protection against infection caused by a different gram-negative bacteria.

Gram-negative bacteria suitable for use in obtaining siderophore receptor proteins according to the invention, are those capable of producing siderophore receptor proteins when raised under growth conditions of low iron availability. Examples of useful gram-negative bacteria include *Escherichia coli* (serotypes 01a, 02a, and 078), *Salmonella agona, Salmonella blockley, Salmonella enteriditis, Salmonella hadar, Salmonella heidelberg, Salmonella montevideo, Salmonella senftenberg, Salmonella cholerasuis, Salmonella typhimurium, Pasteurella multocida* (serotype A:3,4), *Klebsiella pneumoniae, Pseudomonas aeruginosa*, and the like. These organisms are commercially available from a depository such as American Type Culture Collection (ATCC), Rockville, Md. In addition, such organisms are readily obtainable by isolation techniques known and used in the art. The gram-negative bacteria may be derived from an infected animal as a field isolate, and screened for production of SRPs, and introduced directly into the preferred iron-depleted media for that bacteria, or stored for future use, for example, in a frozen repository at about −20° C. to about −95° C., preferably about −40° C. to about −50° C., in BHI containing 20% glycerol, and other like media.

For producing the siderophore receptor proteins, conditions of low iron availability are created using culture media that lack iron or have been supplemented with an iron chelating agent to decrease iron availability. Suitable culture media for providing low iron availability and promoting production of the siderophore receptor proteins in gram-negative bacteria, include media such as tryptic soy broth (Difco Laboratories, Detroit, Mich.) and/or brain-heart infusion (BHI) broth which has been combined with an iron-chelating agent, for example, α,α'-dipyridyl, deferoxamine, and other like agents. In a preferred embodiment, α,α-dipyridyl is added to a BHI culture media in a concentration of about 1–500 μg/ml, preferably about 50–250 μg/ml, more preferably about 75–150 μg/ml.

The gram-negative bacteria employed to produce a siderophore receptor protein are cultured in the preferred media for that organism using methodologies and apparati known and used in the art, such as a fermenter, gyrator shaker, or other like apparatus. For example, a culture may be grown in a gyrator shaker in which the media is stirred continuously with aeration at about 300–600 rev/minute, for about 15–20 hours, at a temperature and pH appropriate for growth for that organism, i.e., about 35°–45° C. and about pH 7–7.6, preferably pH 6.5–7.5. The bacterial culture is then processed to separate and purify the siderophore receptor proteins from the outer membrane of the bacteria.

The bacterial culture is concentrated, for example, by centrifugation, membrane concentration, and the like. For example, the cell culture may be centrifuged at about 2,450–20,000×g, preferably at about 5,000–16,000×g, for about 5–15 minutes at about 3°–6° C. The supernatant is removed by decanting, suctioning, pipetting and the like, and the concentrated cell pellet is collected and washed in a compatible buffer solution maintained at about pH 7–7.6, such as tris-buffered saline (TBS), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 3-NN-morpholino) propanesulfonic acid (MOPS), and the like. The washed pellet is resuspended and washed in a compatible buffer solution, i.e., TBS, HEPES, MOPS and the like. The cell material is then treated to solubilize the components of the outer membrane by resuspending the pellet in buffer containing about 0.5–10% sodium N-lauroyl sarcosinate, preferably about 1–3%, at about 4°–10° C. for about 15 minutes to about 3 hours, preferably about 30 minutes to about 2 hours, preferably with continuous stirring.

The bacterial cells are then disrupted by sonication, French pressure, grinding with abrasives, glass bead vortexing, and other like methods known and used in the art, preferably at a temperature of about 3°–6° C. The cell homogenate is then centrifuged at about 10,000–20,000×g for about 10–45 minutes, to separate cell debris from the supernatant fraction containing the outer membrane proteins. The supernatant is collected by decanting, suctioning, pipetting, or other like method, and then concentrated, for example, by ethanol precipitation, membrane concentration, propylene glycol precipitation, and other methods known and used in the art. In a preferred method, the supernatant is treated by passing it through a membrane having a molecular weight cut-off of about 1,000–50,000 MW, preferably about 10,000–25,000 MW, to concentrate the protein and allow contaminating proteins smaller than the molecular weight cut-off to pass through the membrane, and to decrease the amount of detergent. Such membranes are commercially available, for example, from Amicon, Danvers, Mass.

The concentrated supernatant is then reconstituted in a compatible buffer, i.e., TBS, HEPES, MOPS, and the like, about pH 7–7.6, which contains a detergent for solubilizing the outer membrane and extracting the siderophore receptor proteins. It was found that the anionic detergent sodium dodecyl sulfate (SDS), when used as a solubilizing detergent alone without a reducing agent such as 2-mercaptoethanol, is particularly effective for extracting a high quantity of the siderophore receptor proteins without denaturing or altering their immunogenicity such that the proteins will function in vivo as effective immunogens to elicit an antibody response against gram-negative bacteria. The buffer solution contains about 0.1–4% SDS (0.2%), preferably about 0.1–2% SDS, preferably about 0.1–2% SDS.

After about 1–10 minutes, the siderophore receptor proteins are separated from the buffer solution by affinity, ion exchange, size exclusinn and other like chromatographic

methods known and used in the art. Preferably, the SRP preparation is separated with a 4% stacking gel on a 12.5% acrylamide reducing gel. The fractions are then combined, concentrated, for example by centrifuging, and precipitated, for example with an alcohol (i.e., ethanol, methanol, acetone), to remove the SDS. The purified proteins may be used immediately to prepare a vaccine, or may be stored for future use through lyophilization, cryopreservation, or other like technique known and used in the art.

The vaccine of the present invention may be used for preventing and eliminating infections of gram-negative bacteria in poultry and other animals, including humans. The vaccine may be delivered to the animal, for example, by parenteral delivery, injection (subcutaneous or intramuscular), sustained-released repository, aerosolization, egg inoculation (i.e., poultry), and the like, by known techniques in the art. For prophylactic and anti-infectious therapeutic use in vivo, the vaccine contains an amount of a siderophore receptor protein to stimulate a level of active immunity in the animal to inhibit and/or eliminate gram-negative bacterial pathogenesis and/or sepsis.

The siderophore receptor proteins are administered in combination with a pharmaceutical carrier compatible with the protein and the animal. Suitable pharmacological carriers include, for example, physiological saline (0.85%), phosphate-buffered saline (PBS), Tris(hydroxymethyl aminomethane (TRIS), Tris-buffered saline, and the like. The protein may also be incorporated into a carrier which is a biocompatible and can incorporate the protein and provide for its controlled release or delivery, for example, a sustained release polymer such as a hydrogel, acrylate, polylactide, polycaprolactone, polyglycolide, or copolymer thereof. An example of a solid matrix for implantation into the animal and sustained release of the protein antigen into the body is a metabolizable matrix, as described, for example, in U.S. Pat. No. 4,452,775 (Kent), the disclosure of which is incorporated by reference herein.

Adjuvants may be included in the vaccine to enhance the immune response in the animal. Such adjuvants include, for example, aluminum hydroxide, aluminum phosphate, Freund's incomplete Adjuvant (FCA), liposomes, ISCOM, and the like. The vaccine may also include additives such as buffers and preservatives to maintain isotonicity, physiological pH and stability. Parenteral and intravenous formulations of the vaccine may include an emulsifying and/or suspending agent, together with pharmaceutically-acceptable diluents to control the delivery and the dose amount of the vaccine.

Factors bearing on the vaccine dosage include, for example, the age and weight of the animal. The range of a given dose is about 25–5000 μg of the purified siderophore receptor protein per ml, preferably about 100–1000 μg/ml preferably given in about 0.1–5 ml doses. The vaccine should be administered to the animal in an amount effective to ensure that the animal will develop an immunity to protect against a gram-negative bacterial infection. For example, for poultry, a single dose of a vaccine made with Freund's Incomplete Adjuvant would contain about 150–300 μg of the purified siderophore receptor protein per ml. For immunizing a one-day of age bird of about 60 grams weight, the bird may be subcutaneously or intramuscularly injected with an about 0.25–0.5 ml dose. For an about 3-week old bird of about 1.5 lbs, the bird may be injected with about 0.25–1 ml dose. A vaccine for immunizing an about 5-lb. piglet against *Salmonella choleraesuis* would contain about 100–5000 μg protein per ml, preferably given in 1–5 ml doses. In each case, the immunizing dose would then be followed by a booster given at about 21–28 days after the first injection. Preferably, the vaccine is formulated with an amount of the siderophore receptor protein effective for immunizing a susceptible animal against an infection by two or more strains or species of gram-negative bacteria that express a siderophore receptor protein.

For boosting the immunizing dose, the booster may be a preparation of whole cells as conventionally used, or a chemically modified cell preparation, among others. For example, a useful booster is a preparation of a modified *E. coli* such as avirulent R-mutants, as for example, *E. coli* J5 (commercially available from ATCC as ATCC #43754; described by Overbeek et al., *J. Clin. Microbiol.* 25:1009–1013 (1987)), or *Salmonella minnesota* (commercially available from ATCC as ATCC #49284; as described by Jousimies et al., *J. Bacteriol.* 119:753–759 (1974), and Makela et al., *J. Bacteriol.* 119:760–764 (1974)) that lack outer oligosaccharide side chains of the lipopolysaccharide (LPS) layer of the outer membrane. Outer oligosaccharide side chains tend to mask SRPs on the cell membrane in such a way that the immune system does not recognize the SRPs and anti-SRP antibody titers are depressed. To enhance the ability of a booster made with intact bacterial cells to elicit an anti-SRP immune response, the cell membrane of the bacteria can be chemically altered to eliminate the interfering oligosaccharide side chains. Boosting with chemically-modified bacteria such as an R-mutant, advantageously provides an anti-SRP antibody titer that is 5–20 times higher than booster made of a non-modified whole cell bacterial preparation, or a natural field challenge.

Although not intended as a limitation of the invention, the mechanism by which immunization with the present vaccine provides protection against gram-negative bacterial infection is believed to be as follows. After an animal has been immunized with the vaccine, upon being challenged with a pathogenic strain of gram-negative bacteria, the body responds by producing humoral antibodies that block the siderophore receptor proteins on the outer membrane of the bacteria. This prevents iron uptake by the cell, which, in turn, eventually starves the bacteria of required iron nutrients. Another mechanism is that humoral antibodies produced in response to the sidcrophore receptor proteins in the vaccine, bind to the siderophore receptor protein on the bacterial membrane to cause activation of compliment (C'). This results in complement-mediated bacteriolysis, or increased opsonization which leads to increased phagocytosis by the mononuclear phagocytic system.

In addition, the efficacy of this vaccine is based on the use of purified siderophore receptor proteins rather than using whole cells. The immune response in animals vaccinated with a purified SRP preparation is about 20 times greater than the immune response to a preparation of whole cell grown under iron-restricted conditions. During gram-negative sepsis, an animal host mounts an immune response to an invading bacteria. Since the major constituent of the cell wall of gram-negative bacteria is made of lipopolysaccharide (LPS), the immune response of an animal is directed to this structure inducing an immunodominant role for the LPS cell wall. Outer membrane proteins such as IROMPs or SRPs that are not dominant proteins on the surface of the bacterial cell wall induce limited immune response resulting in low antibody titers. Thus, the use of a bacterin made of whole bacterial cells grown under iron restriction to express siderophore receptor proteins provides a limited immune response to the siderophore receptor proteins due to competing antigens on the cell surface. By comparison, immu-

nizing an animal with a vaccine made of purified SRPs, there is less antigenic competition and the animal's immune system focuses its response on the receptor proteins. Serological profiles show a significant increase in antibody titer in the SRP-vaccinated group compared to the whole cell-vaccinated group when boosted with whole cell expressing SRP.

Polyclonal antibodies may be raised to the siderophore receptor protein by hyperimmunizing an animal with an inoculum containing the isolated siderophore receptor protein. The blood scrum may be removed and contacted with immobilized siderophore receptor proteins reactive with the protein-specific antibodies. The semi-purified serum may be further treated by chromatographic methods to purify IgG and IgM immunoglobulins to provide a purified polyclonal antibody sera for commercial use.

Monoclonal antibodies reactive with the siderophore receptor protein may be raised by hybridoma techniques known and used in the art. In brief, a mouse, rat, rabbit or other appropriate species may be immunized with a siderophore receptor protein. The spleen of the animal is then removed and processed as a whole cell preparation. Following the method of Kohler and Milstein (*Nature* 256:496–97 (1975)), the immune cells from the spleen cell preparation can be fused with myeloma cells to produce hybridomas. The hybridomas may then be cultured and the culture fluid tested for antibodies specific for siderophore receptor proteins using, for example, an ELISA in which specific siderophore receptor proteins are attached to a solid surface and act as capture antigens. The hybridoma may then be introduced into the peritoneum of the host species to produce a peritoneal growth of the hybridoma, and ascites fluids containing the monoclonal antibody to the bacteria may be collected.

The monoclonal antibodies may be used in diagnostic and therapeutic compositions and methods, including passive immunization. For example, immunoglobulins specific towards a siderophore receptor protein may be used to provide passive immunity against gram negative sepsis. Animals may be treated by administering immunoglobulins intramuscularly at about 100/mg/kg body weight, about every 3–7 days.

A method for diagnosing an infection by gram-negative bacteria in a body sample may be carried out with the polyclonal antibody sera or monoclonal antibodies described hereinabove, in an enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), immunofluorescent assay (IFA), a Northern, Western or Southern blot assay, and the like. In brief, the antibody or body sample (i.e., tissue sample, body fluid) may be immobilized, for example, by contact with a polymeric material such as polystyrene, a nitrocellulose paper, or other like means for immobilizing the antibody or sample. The other antibody or body sample is then added, incubated, and the non-immobilized material is removed by washing or other means. A labeled species-specific antibody reactive with the later is added. The serum antibody or gram-negative bacteria in the body sample, is then added and the presence and quantity of label is determined to indicate the presence and amount of gram-negative bacteria in the body sample.

The invention will be further described by reference to the following detailed examples, wherein the methodologies are as described below. These examples are not meant to limit the scope of the invention that has been set forth in the foregoing description. Variation within the concepts of the invention are apparent to those skilled in the art. The disclosures of the cited references throughout the application are incorporated by reference herein.

EXAMPLE 1

Production and Purification of Siderophore Receptor Proteins

Escherichia coli serotype 078 (turkey isolate; serotyped by Pennsylvania State University, deposited with the American Type Culture Collection (ATCC), Bethesda, Md., U.S.A., as ATCC #55652, on Jan. 3, 1995) 700 ml at 10^8 colonies/ml) was inoculated into a Virtis bench-top fermenter (Virtis, Inc., Gardiner, N.Y.), charged with 20-L of brain-heart infusion (BHI, Difco Laboratories, Detroit, Mich.) containing 50 μgrams/ml of dipyridyl (Sigma Chemical Co., St. Louis, Mo.) at 41° C. This isolate has been shown to produce four siderophore receptor proteins for (MW 89 kDa, 84 kDa, 78 kDa, 72 kDa) under iron-restrictive conditions. The pH was held constant at 7.4 by automatic titration with 5N NaOH. The fermenter was stirred at 400 rpm. The culture was grown continuously for 18 hours after which the bacteria were removed by continuous-flow centrifugation at 20,000xg at 4° C. using a Beckman (Model J2-21M) centrifuge (Beckman Instruments, Eden Prairie, Minn.). The pelletized bacteria were washed two times with 1,000 ml physiological saline (0.85%) to remove contaminating culture media proteins.

The bacteria were resuspended in tris-buffered saline (TBS) containing 2.0% sodium N-lauroyl sarcosinate (SARKOSYL™, Sigma Chemical Co., St. Louis, Mo.), optical density 5%, 540 nm. The suspension was incubated at 4° C. for 45 minutes with continuous stirring. The cells were then disrupted using a continuous-flow cell sonicator (Banson 450, Danbury, Conn.) at 4° C., with a maximum flow rate of 5 gph. The disrupted cell suspension was centrifuged at 16,000xg for 20 minutes.

The effluent from the continuous-flow cell sonicator containing the outer membrane proteins was collected and concentrated using ethanol precipitation at −20° C. It is understood that the supernatant may also be concentrated by membrane concentration using a 50,000 MW cut off diaflow membrane (Amicon, Danvers, Mass.). The concentrated material (10% T at 540 nm) was solubilized using 0.2 percent sodium dodecyl sulfate (SDS) in TBS at pH 7.4.

The elution profile of the concentrated material treated with 0.2% SDS is shown in FIG. 1. The solubilized material was applied to a Vantage column (Amicon, Danvers, Mass.) containing 3.2-L of cellufine fast flow GC-700 gel matrix (Amicon, Danvers, Mass.) equilibrated with TBS containing 0.2% SDS at 25° C. Purification of the protein was monitored by UV absorption at 280 nm. Flow rate through the column was 3,000 ml/hr and 15-ml fractions were collected using a UA-5 Detector and Retriever 5 fraction collector (ISCO, Inc., Lincoln, Nebr.). Fractions from each peak were pooled and concentrated using a Diaflo ultrafiltration apparatus with a 50,000 MWCO membrane. Concentrated material from each peak was examined by gel electrophoresis. As shown in FIG. 1, peak 1 contained approximately 85% pure siderophore proteins. This solution was ethanol precipitated at −20° C. for 24 hours to remove the SDS, and then resuspended in phosphate buffered saline (PBS). The amount of protein was determined using a Pierce BCA protein assay (Pierce, Rockford, Ill.).

EXAMPLE 2

Preparation of Vaccine with Siderophore Receptor Proteins

The precipitate from Example 1, hereinabove, containing siderophore receptor proteins of *E. coli* serotype 078, were

resuspended in physiological saline (0.85%) containing 0.1% formalin as a preservative. The protein concentration was 300 μg/ml. The aqueous protein suspension (1,000 ml) was emulsified in a water-in-mineral oil adjuvant containing 972 ml Drakeol 6 mineral oil and 28 ml of Anlacel A as an emulsifier. The mixture was emulsified using an Ultra-Turrax T50 emulsifier (KIKA Works, Inc., Cincinnati, Ohio) at 4° C. The water-in-oil emulsion was stored at 4° C.

EXAMPLE 3

Vaccination of Poultry with Siderophore Receptor Protein Vaccine

Seventy-two turkey poults were raised in isolation from one day of age. At three weeks of age, the birds were divided into two equal groups. Group 1 was vaccinated subcutaneously with the vaccine from Example 2 above, at a dosage level of 150 μg of siderophore receptor protein per bird. Group 2 remained as non-vaccinated controls. Group 1 was given a booster vaccination with the vaccine at a dosage level of 250 μg siderophore receptor protein per bird at 18 days after the first vaccination.

The vaccinated and non-vaccinated birds were equally divided among four isolation rooms. Rooms A and B contained the vaccinated birds, and Rooms C and D contained the non-vaccinated controls. At seven weeks of age, birds in Groups A and C were challenged subcutaneously with *Salmonella agona* at 1.0×10^8 cfu/bird. At 24, 48, 72, 96 and 120 hours post-challenge, two controls and two vaccinated birds were killed. The spleens were aseptically removed from each bird and individually weighed, and adjusted to 4 grams/spleen, 10 grams/liver. Each sample was then homogenized in sterile physiological saline using a Stomacher Lab Blender, Model 3500 (Seward Medical, London). Serial ten-fold dilutions of each homogenate was plated in duplicate on brilliant sulfur green plates (Difco Laboratories, Detroit, Mich.).

The results show the quantitative clearance of *Salmonella agona* in spleens of SRP-vaccinated and non-vaccinated turkeys (FIG. 2). Time 0 represents the number of bacteria given to each bird. At 24-hours, post-challenge in the vaccinated birds, the level of bacteria were reduced to zero and remained at that level throughout the sampling period. In contrast, the non-vaccinated controls remained positive for the duration of the experiment.

EXAMPLE 4

Cross-Reactivity of Siderophore IROMPS Produced by *Escherichia coli* (Serotype 078)

Hyperimmunized serum produced against purified siderophore receptor proteins was examined for its cross-reactivity to bacteria from different genera and species. Siderophore receptor proteins were produced in the following bacteria: *Escherichia coli* (serotypes 01a, 02a and serotype 078 (ATCC #55652)), *Salmonella agona, Salmonella blockley, Salmonella enteriditis, Salmonella hadar, Salmonella Heidelberg, Salmonella montevideo, Salmonella senftenberg, Salmonella choleraesuis*, and *Pasteurella multocida* (serotype A:3,4; deposited with ATCC as ATCC #55657, on Feb. 14, 1995. These bacteria, except for *S. choleraesuis*, were field isolates obtained from clinically diagnosed birds and serotyped by the State Poultry Testing Laboratory, Willmar, Minn. (*Salmonella spp.*) and Pennsylvania State University (*E. coli*). *Salmonella choleraesuis* was obtained from the University of Minnesota Diagnostic Laboratory. The bacterial isolates were grown in 100 ml of BHI broth with dipyridyl (175 mM), and without dipyridyl but containing 200 μm ferric chloride.

The bacteria were collected from the cell cultures by centrifugation at 16,000xg for 10 minutes at 4° C. The cell pellets were washed twice in tris-buffered saline (TBS) at pH 7.4 and resuspended in 30 ml TBS. The cells were ultrasonically disrupted for 2 minutes at 4° C. using a Branson Ultrasonic Sonicator (Danbury, Conn.). The disrupted cell suspension was centrifuged at 16,000xg for 20 minutes at 4° C. The supernatant was collected centrifuged at 30,000xg for 2 hours at 4° C. The pellet was resuspended in 10 ml TBS containing 2% sodium n-lauroyl sarcosine and placed on a gyratory shaker for 45 minutes at 4° C. The detergent insoluble outer membrane protein-enriched fraction was collected by centrifugation at 30,000xg for 2 hours at 4° C. The pellet was resuspended in 1 ml TBS and stored at −90° C. Proteins were separated by SDS-PAGE with a 4% stacking gel on a 12% resolving gel. Laemmli, U. K., *Nature*, 227:680–685 (1970).

The outer membrane proteins from the different *E. coli*, Salmonella and Pasteurella isolates were transferred from the SDS-PAGE gels to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Calif.). The membranes were probed with negative (control) and positive antisera to the siderophore receptor proteins.

The control antisera was collected from the birds in group 2, as described in Example 3 hereinabove. The positive antisera was collected from birds in group 1 from Example 3 hereinabove, at 5 days after the second vaccination. The sera, 50 ml each, were absorbed with killed whole cell bacteria (*E. coli* 078, *Salmonella heidelberg, Pasteurella multocida*) grown in iron-replete media (BHI containing 200 μm ferric chloride) for 1 hour at 4° C.

The SDS-PAGE patterns of the outer membrane protein extracts of the different bacterial isolates, showed expression of siderophore receptor proteins when grown under conditions of iron restriction, in contrast to non-iron restricted controls which did not express siderophore receptor proteins. *Pasteurella multocida* produced three siderophore receptor proteins under conditions of iron restriction which had molecular masses of approximately 96 kDa, 84 kDa and 80 kDa. The *E. coli* isolates produced slight variation in their IROMP profiles. Serotype 078 produced four siderophore receptor proteins with approximate molecular mass of 89 kDa, 84 kDa, 78 kDa and 72 kDa. Serotype 02a produced three bands with molecular weights of 89 kDa, 78 kDa and 72 kDa. Serotype 01a produced two bands with molecular weights of 84 kDa and 78 kDa. All of the Salmonella isolates examined produced three siderophore receptor proteins with identical banding patterns with approximate molecular weights of 89 kDa, 81 kDa and 72 kDa.

Western blot analysis revealed that the positive antisera prepared against the purified siderophore receptor proteins of *E. coli* 078 reacted intensely with the siderophore receptor proteins of *E. coli* serotypes 01a, 02a and the receptor proteins of Salmonella. The 96 kDa and 84 kDa receptor protein of Pasteurella reacted with the positive *E. coli* protein antisera. These results show that the siderophore receptor proteins of *E. coli* have complete antigenic homology to Salmonella and partial homology to *Pasteurella multocida*. The control sera did not react with any siderophore receptor proteins of those species.

EXAMPLE 5

Cross-Reactivity of Siderophore Receptor Proteins of *Escherichia coli* (Serotype 078)

Escherichia coli isolates (150 isolates) originating from colisepticemic birds were screened for reactivity with the

positive antisera of Example 4, hereinabove. The isolates were examined by direct agglutination using the siderophore receptor antisera and negative reference sera. Ninety-eight percent (98%) of the *E. coli* isolates were agglutinated using the positive antisera in contrast to the negative sera. The positive antisera also reacted with *Pseudomonas aeruginosa, Klebsiella pneumoniae* and five sero groups of Salmonella (serotype B, C_1, C_2, D_1 and E_4).

EXAMPLE 6

Serological Response to Siderophore Receptor Proteins (SRP) of *E. coli* in Vaccinated and Non-Vaccinated Flocks Under Natural Field Conditions

Fifty one thousand, one-day old turkey poults were equally divided among two barns designated as barns 1 and 2. At six weeks of age, birds in barn 1 were subcutaneously injected with a water-in-oil vaccine as described hereinabove in Example 2. Each bird received 0.5 cc containing 300 μg *E. coli* serotype 078 siderophore receptor protein (SRP) in the lower neck region. Barn 2 remained as non-vaccinated controls. Blood was drawn from 15 birds per barn at weekly intervals.

FIG. 3 represents the serological response to *E. coli* SRPs between vaccinated and non-vaccinated flocks. The antibody response to the SRPs in the vaccinated flock increased steadily with each sampling period as compared to non-vaccinated controls. At 35 days following vaccination, the vaccinated group had a 7.1 times greater antibody response than the control group.

Table 1, below, shows the average weight of processed birds between the vaccinated and non-vaccinated flocks. There was a statistically greater weight advantage between the vaccinated flock (12.2 lbs/bird) as compared to the non-vaccinated flock (11.8 lbs/bird).

TABLE I

THE AVERAGE BODY WEIGHT BETWEEN SRP-VACCINATED AND NON-VACCINATED TURKEYS AT TIME OF PROCESSING

Barn 2 (non-vaccinated)		Barn 1 (SRP-vaccinated)	
#of Birds/lot	Ave. Body weight (Lbs)	#of Birds/lot	Ave. Body weight (Lbs)
2772	11.85	1986	12.00
3108	11.91	3168	12.11
3024	11.92	3072	12.04
3168	11.97	3060	12.25
3256	11.98	3072	12.36
3186	11.75	3072	12.57
3136	11.65	3024	12.31
2112	11.42	3024	12.16
Total 23762	Mean 11.8 SD 0.192 CV 1.63	Total 23460	Mean 12.2 SD 0.18 CV 1.54

FIGS. 4 and 5 show the total percent mortality and culls in *E. coli* SRP-vaccinated sister flocks (i.e., originating from the same breeder hens or hatchmates), and the non-SRP-vaccinated controls, from 3–13 weeks of age. These results show the true field mortality after vaccination, by excluding early poult mortality which could result in erroneous results. As can be seen, there was a significant reduction in both mortality and birds culled in the SRP-vaccinated flocks. These results demonstrate the usefulness of *E. coli*-derived siderophore receptor proteins in a vaccine for controlling systemic infections caused by *E. coli* under natural field conditions.

EXAMPLE 7

Cross-Reactivity of SRPs of *Salmonella senftenberg* and *Pasteurella multocida*

Forty-eight Nicholas turkey poults were raised in isolation from one day of age. At three weeks of age, the birds were divided into two equal groups designated as Group 1 and Group 2. Twelve birds in Group 1 were vaccinated subcutaneously with (0.5 cc) 300 μg purified SRP isolated from *Salmonella senftenberg*. The vaccine was prepared as described in Example 2 above. The remaining twelve birds were used as non-vaccinated controls. Birds in Group 2 were treated the same as in Group 1, except 12 of the birds were vaccinated with 300 μg purified SRP isolated from *Pasteurella multocida*.

Blood was taken from all of the birds in both groups at 5 day intervals. Fifteen days after the first injection, vaccinated birds received a second injection of the appropriate SRP. Each vaccinated bird received 500 μg, (0.5 cc) SRP subcutaneously in a water-in-mineral adjuvant. All non-vaccinated birds remained as controls. Birds were bled at 5-day intervals.

Fifteen days after the second injection, the vaccinated birds in Group 1 were intravenously challenged with 100 μg *S. heidelberg* SRP (FIG. 7). Blood was taken at 2-day intervals post challenge. There was a high antibody response to challenge at 2- and 4-days post challenge. This data shows the cross-reactivity of *S. heidelberg* to *S. senftenberg*. These proteins, in turn, both cross-react with *E. coli*, as demonstrated by the ELISA using *E. coli* SRPs as the capture antigen according to the protocol described hereinabove in Example 5.

Likewise, 15 days after the second injection, all birds in Group 2 were challenged intramuscularly with 1.1×10^6 CFU of *P. multocida*, ATCC strain P-1059. Mortality was recorded daily for 2 weeks post-challenge. FIG. 6 and Table 2 below also shows the mortality between the vaccinated and non-vaccinated birds following challenge.

TABLE 2

Mortality of Vaccinated and Non-Vaccinated Turkeys Following Challenge with *Pasteurella multocida* P-1059

	Numbers of dead/total tested	
	Non-vaccinated	Vaccinated
	11/12 (91.6%)	1/12 (8.3%)

Eleven (91.6%) of the non-vaccinated birds died within 14 days after challenge (see, FIG. 6). In contrast, only 1 (8.3%) of the birds in the vaccinated group died. These results demonstrate that siderophore receptor proteins can be used as protective immunogens.

FIGS. 7 and 8 show the serological response of birds vaccinated with siderophore receptor proteins isolated from *S. senftenberg* and *P. multocida*, respectively. The siderophore receptor proteins induced primary and secondary immune responses in both vaccinated groups at 10 and 20 days post-vaccination as compared to non-vaccinated control birds. These antibody responses demonstrate the cross-reactive nature of these protein, which was confirmed in the ELISA assay using SRPs isolated from *E. coli* as capture antigens.

EXAMPLE 8

Cross-Reactivity of Siderophore Receptor Proteins as Evaluated by ELISA

The cross-reactivity of *E. coli* siderophore receptor proteins from Example 7 above was further examined using an

Enzyme-Linked Immunosorbent Assay (ELISA). The siderophore receptor proteins (SRPs) were purified from polyacrylamide gels using a model 422 electro-eluter (Bio-Rad Laboratories, Hercules, Calif.). The proteins were then used as capture molecules in an indirect ELISA test.

The optimum working concentrations of SRP and conjugate was determined by several chequerboard titrations using positive and negative control serums from Example 6 above. A prediction curve was then established to calculate SRP ELISA titers at a 1:200 dilution. All subsequent tests were performed at a single serum dilution (1:200) and SRP titers were calculated from the average of duplicate test absorbance values.

The ELISA was performed by adding 100 μl of diluted SRP of *E. coli* in 0.05M (0.1 ug) carbonate buffer (pH 9.6) to each well of a 96-well flat-bottom, easy wash microtiter plate (Corning, Corning, N.Y.). After overnight incubation at 4° C., excess SRP was removed and the plate was washed. All subsequent washing steps were done three times in phosphate-buffered saline (pH 7.4) with 0.05% Tween 20. The plates were blocked for one bour at 37° C. with 4% Fisb Gelatin (Sigma) in PBS and then washed.

Duplicate serum samples from Example 7 were tested in parallel at single-point dilutions using 100 μl/well and incubated for 40 minutes at 37° C. Each plate contained positive and negative control sera obtained from birds from Example 4 above. After washing, 100 μl peroxidase-labeled conjugate was added to each well. After incubation for 40 minutes at 37° C., the plates were washed and 100 μl of ABTS peroxidase substrate in buffered H_2O_2 solution (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) was added to each well. The substrate was allowed to react for 15 minutes at room temperature. The reaction was terminated with 50 μl of 1% SDS and the absorbance read directly using a MR650 microtiter plate reader (Dynatech Laboratories, Alexandria, Va.).

EXAMPLE 9

Fermentation protocol for production of siderophore receptor proteins

The following protocol was used to culture *E. coli* 078 (ATCC #55652) resulting in expression of six (6) siderophore receptor proteins.

An *E. coli* master seed stock was prepared by growing the organism in 2000 ml of sterile BHI broth containing 1–500 μg 2,2'-dipyridyl for 8 hours at 37° C. The bacteria were harvested by centrifugation at 10,000xg for 30 minutes. The culture is washed twice by centrifugation and resuspending the pellet in sterile PBS. The final pellet was resuspended into 500 ml sterile BHI containing 20% sterile glycerol. One milliliter of culture was transferred to a 2-ml cryovial and stored at −85° C.

A cryovial (1 ml) of the *E. coli* master seed stock was used to inoculate a 100-ml culture flask containing tryptone (10 g/l), yeast extract (5 g/l), dextrose (2 g/l), NaCl (10 g/l), and 2,2'-dipyridyl (15.0 μg/ml). The culture was incubated at 37° C. for 7 hours, at which time it was inoculated into 2 liters of the above media and allowed to grow for an additional 4 hours at 37° C. The 2-liter culture was used to inoculate a 20-liter Virtis bench-top fermenter (Model 233353, Virtis, Gardiner, N.Y.) charged with 13 liters of tbe above-described media. The pH was held constant between 6.9 and 7.2 by automatic titration with 30% NaOH and 10% HCl. The stirring speed was 250 rev/minute, and the culture was aerated with 11 liters/minute at 34° C. Foaming was controlled automatically by the addition of 0.4% silicone defoamer (Antifoam-B, J. T Baker, N.J.). The culture was allowed to grow continuously at these conditions for 12 hours (O.D. 600 nm=7.10) at wbich time it was pumped into a 150-liter fermenter (W. B. Moore, Easton Pa.) charged with 110 liters of the above-described media containing 26.7 μg/ml dipyridyl and 0.2% defoamer. The conditions in the fermenter were as follows: 450 rpm, 50 slpm air, 10 psi backpressure, 34° C., and pH held at 6.9 with NaOH.

After 12 hours of fermentation, tbe bacteria were inactivated by the addition of 0.15% formalin. The bacteria were harvested by continuous flow centrifugation (20,000xg at 4° C.) using two Beckman (Model J2-21M) centrifuges equipped with JCF-Z continuous flow rotors.

The pelletized bacteria were then washed to remove contaminating culture media proteins and furtber processed as described above in Example 1. The concentrated material was treated with 0.2% SDS and eluted as described above in Example 1. The peak from the elution profile containing approximately 85% pure siderophore receptor proteins was ethanol precipitated to remove SDS, and resuspended in PBS.

The material was separated by SDS-PAGE as described above in Example 4 with a 4% stacking gel on 12.5% acrylamide gel. The SDS-PAGE pattern of the outer membrane protein extract showed expression of SRPs baving molecular weigbts of 91–92 kDa, 89 kDa, 84 kDa, 78 kDa, 74 kDa and 72 kDa.

EXAMPLE 10

Efficacy of vaccine of SRPs from *Escherichia coli* under natural field conditions

The efficacy of vaccinating turkeys with *E. coli* siderophore receptor proteins (SRPs) under natural field conditions was shown as follows. A farm complex with a history of disease was chosen for experimental trials. The facility was a three state operation, having two brooding barns and eight finishing farms.

Data was collected for one year prior to vaccination to establish an accurate profile on mortalities and bird performance (flocks 1–16 before vaccination). Vaccinating with SRPs was evaluated for a period of 6 months (flocks 17–24 after vaccination). A total of 24 flocks comprising 1,160,864 birds was examined. Vaccination trials began in January and ran through July, considered to be a critical time period for *E. coli* infections and other natural field challenges.

Brooder barns 1 and 2 were divided in half and designated as A and B (barn-1) and C and D (barn-2). Approximately 50,000 randomized hens were placed in each barn so tbat each flock contained 25,000 birds. All flocks were vaccinated by subcutaneous injection at 3 weeks of age with a vaccine preparation containing SRPs (MW 91–92 kDa, 89 kDa, 84 kDa, 78 kDa, 74 kDa and 72 kDa, SDS-PAGE on 12.5% acrylamide gel) isolated and purified from *E. coli* 078 as described above in Example 1. Flocks A and C were vaccinated with a dosage level of 300 μg SRP and 10^9 $TCID_{50}$ Newcastle Disease Virus (NDV) in a water-in-oil emulsion. Flocks B and D were the controls, and given a dosage level of 10^9 $TCID_{50}$ NDV only.

At 4 weeks of age, the birds were moved into four second-stage barns while maintaining identity. At nine weeks of age, the birds were moved to four finishing barns, keeping identity on each 25,000 bird flock. Birds were marketed at 12- and 14-weeks of age and identity was maintained throughout processing.

Table 3 shows the cumulative farm history before and after SRP-vaccination. Twenty-four flocks were evaluated, the 16 before vaccination (1–16) and the 8 vaccinated flocks (17–24) including controls. Flocks 1–16 were not SRP-vaccinated and included as a farm history to show the performance advantage to SRP-vaccinated flocks 17–24.

Table 3 below, shows the age at which each flock was marketed, the head count, total percent mortality, condem (i.e., condemnation at processing), and average bird weight/lot processed.

TABLE 3

Flocks	Age (days)	Head Count	Mortality (%)	Condem (%)	Ave. wt.
Flock History Before SRP-vaccination					
1	97	47818	8.37	1.13	13.88
2	94	45638	12.53	1.17	13.80
3	95	51443	12.87	3.44	13.58
4	96	49999	4.20	1.23	13.86
5	92	49733	4.68	0.96	13.25
6	96	48303	7.36	1.25	13.49
7	101	48722	16.50	2.12	15.10
8	103	51456	12.26	1.41	15.60
9	98	50423	7.84	1.63	14.73
10	96	50880	7.04	1.59	13.81
11	95	46710	14.85	1.16	14.04
12	98	48994	11.32	1.09	13.89
13	92	43433	21.28	1.74	13.23
14	94	49806	9.59	1.08	13.64
15	93	39216	28.08	2.35	12.92
16	96	46119	15.95	1.45	13.76
Flock History After SRP-vaccination					
17	99	48323	8.08	1.45	15.37
18	96	48091	8.15	1.16	14.93
19	96	48748	6.89	1.07	16.16
20	90	48462	7.36	1.06	14.11
21	92	49175	6.11	1.00	15.08
22	90	48261	7.86	0.83	14.38
23	94	51813	5.95	0.92	15.52
24	98	49295	9.44	1.08	16.10
Mean	96/94	48043/49021	12.2/7.5	1.6/1.07	13.9/15.3
SD	2.9/3.5		6.2/1.2	0.63/0.18	0.70/0.77
CV	3.1/3.7		51.4/15.5	40.9/17.2	5.0/5.0

As shown above in Table 3, the average percent mortality before vaccination was 12.2±6.2 with a coefficient of variation (cv) of 51.4% as compared to the average mortality after vaccination of 7.5±1.2 with a cv of 15.5%. This is a 4.7% decrease in mortality, which equates to 4700 birds for every 100,000. The decrease in the coefficient of variation (51.4% as compared to 15.5%) on total mortality illustrates a positive effect on bird livability and uniformity. FIG. 9 is a graphical representation of mortalities in consecutive flocks before and after vaccination.

Condemnation was also positively effected sbowing 1.6±0.63 percent before vaccination as compared to 1.07±0.18 percent after vaccination (Table 3 above). The difference, 0.53% is significant considering the number of birds processed.

A dramatic effect that was observed by the SRP vaccination was tbe increased weight advantage, as seen above in Table 3. Before vaccination the average bird weight was 13.9±0.70 pounds, with an average growing time of 96 days. The average weigbt per bird after vaccination was 15.3±0.77 pounds, with an average growing time of 94 days. These results demonstrate tbe advantage in performance that can be obtained through SRP-vaccination.

FIG. 10 shows the serological response to SRPs of *E. coli* between tbe SRP-vaccinated and non-SRP-vaccinated flocks as determined by ELISA, using purified *E. coli* SRPs as the capture molecule. The assay was conducted as described above in Example 8. The profile was consistent between the vaccinated and non-vaccinated flocks under natural field conditions. As the profile illustrates, once the bird's immune system becomes focused to recognize these proteins, continuous field challenge by bacteria expressing SRPs causes a steady rise in antibody titer to a level which provides protection and/or to the point where systemic challenge does not effect performance.

Using purified IROMPs in a vaccine optimizes the animal's immune system to focus on those proteins. The birds vaccinated with 300 μg purified SRP at three weeks of age showed an increase in titer at 11 weeks of age which was 10,000 times greater than tbe titer in tbe non-SRP-vaccinated controls. This increase in titer is the result of focusing the immune system to recognize these proteins. Once vaccinated, the bird establishes a population of memory cells tbat are activated upon cacb field challenge. Under natural field conditions, tbe bird is continuously challenged by gram-negative bacteria sucb as *E. coli*, whicb express SRPs that cross-react and cause a continuous rise in antibody titer (as was seen in the SRP-vaccinated birds). By comparison, the control birds under the same conditions, sbow low antibody titers even though exposed to the same field challenges.

EXAMPLE 11

Vaccination with SRP-vaccine and vaccine made with bacterial whole cells

A comparison was made between turkeys injected with a vaccine made of purified SRPs derived from *Salmonella heidelberg* prepared as described above in Example 1, and a vaccine made of bacterial whole cells of tbe same organism grown under iron-restrictions so as to express SRP on tbe cell surface. The wbole cell bacteria was prepared as described in Example 1, except for the following modification: after the fermentation process 0.3% formalin were added to the vessel to kill the organism. The killed bacteria were collected as described in Example 1, washed and resuspended in physiological saline, and adjusted to an optical density of 35% T at 540 mm to give approximately 10^7 bacteria/ml. The vaccine was prepared as described above in Example 2.

Forty-five tbousand one-day old bybrid turkey poults (bens) were raised to 4 weeks of age, on a brooding facility. At four weeks of age, tbe birds were moved to a growing facility and equally divided among two barns designated as barns 1 and 2. At 6 weeks of age, birds in barn 1 were vaccinated subcutaneously in the lower neck with 0.5 cc of the SRP vaccine while the birds in barn 2 were vaccinated with the wbole cell preparation. Blood was taken from 12 birds/barn at weekly intervals to monitor tbe serological response to SRP between the two groups.

FIG. 11 shows tbe titer to SRP between wbole cell and SRP-vaccinated birds. The immunological response to SRP was significantly greater in purified SRP-vaccinated group as compared to the wbole cell vaccinated group. These results clearly demonstrate the efficacy of using a substantially pure preparation of SRP for inducing an immune response in an animal in contrast to using whole cell expressing the same SRP.

EXAMPLE 12

Transfer of anti-SRP antibodies to Breeder Hen Progeny

The 10-day mortality in progeny from SRP-vaccinated and non-vaccinated breeder bens was evaluated to assess the transfer of anti-SRP antibodies from adult to progeny.

Twenty thousand randomized Nicholas turkey poults (hens) were equally divided among two brooder barns designated as barns 1 and 2. At four weeks of age, all birds in barn 1 were vaccinated with 300 μg of *E. coli* SRP and Newcastle Disease Virus (NDV) in a water-in-oil vaccine. Birds in barn 2 were given NDV only and acted as controls. At 24 weeks of age, the birds from barn 1 were given a second injection of SRP at 300 μg/bird. Birds from barn 2 remained as non-vaccinated controls. At thirty weeks of age, the birds were placed in barns 1 and 2 of a laying farm. At mid-lay, eggs were collected from the SRP-vaccinated and non-vaccinated hens. Eggs were set in separate incubators and hatchers. At hatch time, all poults were treated the same and identity was maintained throughout sexing and servicing.

Five thousand poults (hens) from each group were placed in a commercial brooding barn and kept in brooding rings at 7 rings/group containing 714 poults/ring. Poult mortality was monitored for each ring/group for a period of 10 days.

The total 10-day mortality in poults originating from the SRP-vaccinated hens was 105 (2.1%) as compared to 160 (3.2%) in the non-vaccinated progeny (FIG. **12**). This is a 1.1% advantage in poult livability, which equates to 1100 poults for every 100,000. This is significant considering that there are 200 million turkeys in the United States and 7 billion broilers worldwide.

These results show the beneficial effect of vaccinating breeding stock to induce maternal antibody to SRP in progeny to reduce gram-negative infections that are responsible for much of the early poult mortality.

EXAMPLE 13

Cross-reactive and cross-protective nature of siderophore receptor proteins (SRP) between different serogroups of salmonella

The SRP of *Salmonella enteritidis* (Se), serogroup D_1 and *Salmonella typhimurium* (St), serogroup B were examined for their ability to cross-react and cross-protect. Briefly, 160 randomized hybrid turkey poults (hens) were raised in isolation. At three weeks of age, the birds were equally divided among 4 isolation rooms, 40 birds/room, designated as A, B, C and D. Birds in group C were subcutaneously injected with a water-in-oil vaccine, as described hereinabove in Example 2, containing 300 μg SRP of *S. typhimurium*. Birds in room D were subcutaneously injected with 300 μg SRP of *S. enteritidis*. Birds in rooms A and B remained as non-vaccinated controls. Blood was taken from 10 birds/group at weekly intervals to monitor the serological response to SRP.

Twenty one days after the first injection, birds in groups C and D were given a second injection containing 300 μg of the appropriate SRP. Blood was taken at 5 and 10 days after the second injection. The serological response to SRP was examined by ELISA using *E. coli* SRP as the capture molecule as described above in Example 8.

FIGS. **13** and **14** show the serological response of birds vaccinated with SRP isolated from *S. typhimurium* and *S. enteritidis*. The immunological response to SRP increased steadily in both groups with each sampling period as compared to the non-vaccinated controls, showing the immunogenicity of these proteins. Importantly, these results show the cross-reactive nature of these proteins since the ELISA is using *E. coli* SRP as the capture molecule.

Fifteen days after the second injection, all birds were intravenously challenged with a nalidixic acid resistant strain of *S. enteritidis* or *S. typhimurium* at 5.0×10^7 colony forming units (CFU)/bird. These bacteria were made resistant to nalidixic acid to enhance their isolation by incorporating nalidixic acid in the recovery media which eliminated any contamination. Bacteria resistant to nalidixic acid were prepared as follows: One ml of a 12-hour Tryptic soy broth (TSB) culture of *S. enteritidis* and/or *S. typhimurium* containing approximately 10^8 viable organisms, was spread over the surface of a brilliant sulfur green (BSG) agar (Difco) plate containing 500 μg/ml nalidixic acid (Sigma). The plates were incubated at 37° C. for 24 hours and the colonies that grew were cloned by plating on BSG containing 250 μg/ml nalidixic acid. The nalidixic acid-resistant strains of salmonella were incubated in 100 ml of TSB at 37° C. for 12 hours At the end of incubation, the culture was centrifuged (10,000×g) and washed twice in PBS (pH 7.4), and the optical density was adjusted to 35% transmission at 540 nm to obtain 5.0×10^7 CFU/ml. These isolates were then used for challenge.

To evaluate homologous and heterologous protection, twenty birds in room C (vaccinated with St-SRP) were wing banded and moved into room D, and 20 birds in room D (vaccinated with Se-SRP) were wing banded and moved to room C. All birds in room C (20 St-vaccinated and 20 Se-vaccinated) were challenged with *S. typhimurium*, while birds in room D (20 Se-vaccinated and 20 St-vaccinated) were challenged with *S. enteritidis*.

At 24, 48, 72 and 96 hours post-challenge, two birds from each group were killed. The spleens were aseptically removed from each bird and individually weighed, and adjusted to 4 grams/spleen. A fecal sample from the cecal junction from each bird was also taken. Each sample was weighed and adjusted to 0.5 grams. Four milliliters of sterile saline was added to each spleen and 0.5 ml to each fecal sample. Each sample was homogenized using a Stomacher Lab Blender (Sewert Medical, London) for 1 minute. Serial ten-fold dilutions of each homogenate were plated in duplicate on BSG plates containing 250 μg/ml nalidixic acid.

The results show the quantitative clearance of *S. typhimurium* (St) (FIG. **15**) and *S. enteritidis* (Se) (FIG. **16**) in spleens of SRP-vaccinated and non-vaccinated turkeys. As shown in FIGS. **15** and **16**, there was a steady decline in the number of bacteria/spleen. At 96 hours after challenge (cblg), the difference between the vaccinated and non-vaccinated groups was approximately 2.5 logs. An important aspect of these results is the cross-protective nature induced by these proteins. FIG. **15** shows the cross-protective nature of the birds vaccinated with the SRP of Se but challenged with St. FIG. **16** shows this same cross-protective effect of birds vaccinated with SRP of Se and then challenged with St. All vaccinated groups showed a significant reduction in the number of bacteria in spleens in contrast to the non-vaccinated birds.

At 72 and 96 hours after challenge, intestinal shedding of Salmonella was detected in the non-vaccinated birds at greater then log 4. In contrast, all of the vaccinated birds were negative for Salmonella within this same sampling period. These results indicate that these proteins may have some beneficial effect in preventing the intestinal colonization of Salmonella.

EXAMPLE 14

Preparation and use of the 37–38 kDa transmembrane and porin proteins in a vaccine

The transmembranes and porin proteins (MW 34–38 kDa), identified as OmpA, OmpC, OmpD and OmpF are

expressed with and without iron. These proteins can be purified as described above in Example 1, by collecting fractions 1650–2250 as shown in FIG. 1. These proteins can be combined with peak 1 (FIG. 1) to obtain a combination of SRP and porin proteins that are conserved among Salmonella, *E. coli*, and Pasteurella.

A vaccine containing *E. coli* SRPs (MW 89 kDa, 84 kDa, 78 kDa and 72 kDa) was combined with porins (MW 34 kDa–38 kDa) to give a total protein content of 600 μg/ml, and prepared as described above in Example 2. The vaccine was used to induce hyperimmunized sera. Briefly, six (6) three-week old turkeys were given a single subcutaneous injection in the lower neck region followed by a second injection 15 days after. Serum was collected 10 days after the second injection.

Western blot analysis, as described above in Example 4, using sarcosine cell wall extracts of *E. coli*, Salmonella and Pasteurella and probed with the above sera revealed cross-negative proteins in the 34 kDa and 38 kDa region as well as the SRPs from each isolate examined.

These results indicate the potential of using conserved protein (SRP and porins) as an effective method for vaccinating against gram-negative infections.

What is claimed is:

1. A vaccine for use in immunizing an animal against an infection by a gram-negative bacteria, said vaccine comprising:

(a) at least four siderophore receptor proteins extracted from an outer membrane of at least two different serotypes of bacteria of the Pasteurellaceae family;

(b) a non-iron-regulated outer membrane protein extracted from a bacteria selected from the group of bacteria in the families of Enterobacteriaceae and Pasteurellaceae and, having a molecular weight of about 34–38 kDa; and

(c) a physiologically acceptable carrier.

2. The vaccine according to claim 1, comprising 5–15 siderophore receptor proteins.

3. The vaccine according to claim 1, comprising a siderophore receptor protein reactive with a siderophore selected from the group consisting of aerobactin siderophore, coprogen siderophore, enterochelin siderophore, citrate siderophore, multocidin siderophore, and ferrichrome siderophore.

4. The vaccine according to claim 1, effective to stimulate production of antibody to the siderophore receptor protein in an adult animal and subsequent transfer of said antibody to progeny of the adult.

5. The vaccine according to claim 1, wherein the carrier is a liquid, and the amount of the siderophore receptor protein in the vaccine is about 25–5000 μg/ml.

6. The vaccine according to claim 5, wherein the carrier is selected from the group consisting of physiological saline, phosphate-buffered saline, Tris(hydroxymethyl aminomethane), and Tris-buffered saline.

7. The vaccine according to claim 1, wherein the carrier is in the form of a solution, water-in-oil emulsion, liposomes, or a metabolizable solid matrix.

8. The vaccine according to claim 1, further comprising an adjuvant selected from the group consisting of aluminum hydroxide, aluminum phosphate, and Freund's Incomplete Adjuvant.

9. The vaccine according to claim 1, wherein the siderophore receptor protein is capable of stimulating production of an antibody reactive with a fungus selected from the group consisting of *Aspergillus flavus, Penicillium spp.* and *Fusarium spp.*

10. The vaccine according to claim 1, wherein the siderophore receptor proteins are obtained from a population of bacteria isolated from a population of animals.

11. The vaccine according to claim 10, wherein the siderophore receptor proteins are derived from 3–200 field isolates.

12. The vaccine according to claim 1 wherein the siderophore receptor proteinS have molecular weight of about 80–96 kDa.

13. The vaccine according to claim 12, wherein the siderophore receptor protein extracted from the bacteria of the Pasteurellaceae family has a molecular weight of about 96 kDa, 84 kDa, or 80 kDa as determined by SDS-PAGE.

14. A method for immunizing an animal against an infection by a gram-negative bacteria, comprising:

(a) administering to the animal a vaccine comprising:

(i) at least four siderophore receptor proteins extracted from an outer membrane of at least two different serotypes of bacteria of the Pasteurellaceae family;

(ii) a non-iron-regulated outer membrane protein extracted from a bacteria selected from the group of bacteria in the families of Enterobacteriaceae and Pasteurellaceae and, having a molecular weight of about 34–38 kDa; and

(iii) a physiological acceptable carrier.

15. The method according to claim 14, wherein the vaccine is administered by subcutaneous injection, intramuscular injection, sustained release repository, aerosolization, or inoculation into an egg.

16. The method according to claim 14, wherein the vaccine induces an effective antibody titer to prevent or eliminate the infection without administration of a booster of the vaccine.

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(54) *IN OVO* VACCINATION AGAINST COCCIDIOSIS

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(57) **ABSTRACT**

The invention relates to a method of vaccinating a domesticated bird against coccidiosis comprising administering in ovo an effective immunizing dose of live Eimeria sporozoites or merozoites, or a mixture thereof. In a preferred embodiment, the domesticated bird that is vaccinated is a chicken or turkey.

16 Claims, No Drawings

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IN OVO VACCINATION AGAINST COCCIDIOSIS

This application is a national stage filing under 35 U.S.C. 371 of PCT/IB95/0046, filed Jun. 7, 1995.

BACKGROUND OF THE INVENTION

The present invention relates to a method of vaccinating domesticated birds against coccidiosis. In particular, the invention relates to the in ovo administration of live Eimeria spp sporozoites or merozoites, or mixtures thereof, into the developing eggs of domesticated birds in order to immunize the hatched chicks against coccidiosis.

Coccidiosis is an enteric disease of domesticated birds caused by infection with intracellular protozoan parasites of the genus Eimeria. Coccidiosis is the most economically devastating parasitic disease of domesticated birds. It is estimated that anticoccidial medications and losses due to coccidiosis cost the poultry industry hundreds of millions of dollars every year.

Various attempts to vaccinate domesticated birds against coccidiosis have been reported since the early 1950's. Current vaccination methods include administering live Eimeria oocysts to birds through feed or water. These methods, however, are inconvenient and inefficient because not all birds get the intended oocyst dose and many are either unprotected by the vaccine or receive a pathogenic infection.

In J. M. Sharma and B. R. Burmester, Avian Dis. 26: 134–149, 1981, the authors reported that chickens vaccinated in ovo with herpesvirus of turkey developed immunity against subsequent challenge with Marek's disease virus. In European patent publication no. 291173, an immunization process is referred to wherein a nonreplicating immunogen is administered in ovo. The immunogens specifically referred to in the European patent are a genetically engineered Eimeria antigen and an Eimeria oocyst extract. The European patent specifically excludes live parasite stages such as those used in the vaccination method claimed herein.

The present vaccination method involves in ovo administration of live Eimeria sporozoites or merozoites, or a mixture thereof, into the developing eggs of domesticated birds. The available literature suggests that such a vaccination method would be ineffective in ovo and should be applied post-hatch. In T. K. Jeffers and G. E. Wagenbach, J. Parasit. 56(4): 656–662, 1970, the authors reported that in ovo injection of *E. tenella* sporozoites on day 10 of incubation provided no significant immunological protection against subsequent challenge with *E. tenella* oocysts. In fact, they reported that chicks that received no treatment had a greater survival rate against subsequent challenge with *E. tenella* oocysts than chicks that had been treated in ovo with sporozoites. In K. L. Watkins et al., Proc. VI th. International Coccidiosis Conf., Abstract E1–2, Ontario, Canada, 1993, the authors described in ovo inoculation with live *E. maxima* sporocysts and sporulated oocysts and concluded that their study provided no evidence that in ovo exposure protects against subsequent coccidial challenge with *E. maxima* oocysts 10 days post-hatch. Watkins et al. further concluded that significant immunological protection is provided if inoculation is done soon after hatch rather than in ovo. Contrary to this teaching, the in ovo vaccination method of the present invention provides unexpected immunity that protects the hatched birds against subsequent coccidial challenge.

SUMMARY OF THE INVENTION

The present invention, also referred to herein as the "present vaccination method", relates to a method of vaccinating a domesticated bird against coccidiosis comprising administering in ovo, during the final quarter of incubation, an effective immunizing dose of live Eimeria sporozoites or merozoites, or a mixture thereof.

The term "domesticated bird(s)", as used herein, unless otherwise indicated, includes chickens, turkeys, ducks, game birds (including, but not limited to, quail, pheasants, and geese) and ratites (including, but not limited to, ostrich).

The term "in ovo", as used herein, unless otherwise indicated, means into a domesticated bird egg containing a live, developing embryo.

The term "administering in ovo" or "in ovo administration", as used herein, unless otherwise indicated, means administering the vaccine described herein to a domesticated bird egg containing a live, developing embryo by any means of penetrating the shell of the egg and introducing the vaccine. Such means of administration include, but are not limited to, injection of the vaccine.

The term "final quarter of incubation", as used herein, unless otherwise indicated, means the final quarter of incubation of a developing egg of a domesticated bird.

The term "Eimeria", as used herein, unless otherwise indicated, means one or more species of the genus Eimeria that infect domesticated birds. Such Eimeria species include those that are found in chicken, including *E. tenella, E. acervulina, E. maxima, E. necatrix, E. mitis, E. praecox,* and *E. brunetti,* and also those that are found in turkeys, including *E, meleagrimitis, E. adenoeides, E. gallopavonis, E. disperse, E. meleagridis, E. innocua,* and *E. subrotunda,* and also Eimeria species that infect other domesticated birds as defined above. The term "Eimeria" also includes all strains of the foregoing species of Eimeria, including, but not limited to, precocious strains, and attenuated strains, which includes strains that have been irradiated, or otherwise treated, so that they fail to complete development. The term Eimeria also includes any newly-discovered strains or species of Elmeria that infect domesticated birds as defined above.

The terms "sporozoites", "sporocysts", "oocysts", and "merozoites", as used herein, unless otherwise indicated, mean live Eimeria sporozoites, sporocysts, oocysts, and merozoites.

The term "effective immunizing dose", as used herein, unless otherwise indicated, means a number of sporozoites or merozoites, or, when mixed, a number of sporozoites and merozoites, sufficient to provide immunological protection in the hatched birds that is greater than the inherent immunity of non-immuized birds. As used herein, the terms "immunize" and "vaccinate" are synonymous and are used interchangeably.

A preferred dose to be administered in accord with the method of the invention comprises 10 to 10^5 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10 to 10^6.

A more preferred dose comprises 10^3 to 10^6 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10^3 to 10^6.

Another preferred dose comprises 10^2 to 10^5 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10^2 to 10^5.

A preferred domesticated bird to be vaccinated in accord with the method of the invention is a chicken.

A preferred dose to be administered in ovo to chicken eggs comprises sporozoites or merozoites, or a mixture thereof, of two or more species of Eimeria selected from tbe group consisting of *E. tenella, E. acervulina, E. maxima, E. necatrix, E. mitis, E. praecox,* and *E. brunetti.* The dose preferably includes from 10 to 10^6 sporozoites or merozoites, or a mixture thereof, for each species that is included in the dose.

Another preferred domesticated bird to be vaccinated in accord with the method of the invention is a turkey.

A preferred dose to be administered in ovo to turkey eggs comprises sporozoites or merozoites, or a mixture thereof, of two or more species of Eimeria selected from the group consisting of *E. meleagrimitis, E. adenoeides, E. gallopavonis, E. dispersa, E. meleagridis, E. innocua,* and *E. subrotunda.* The dose preferably includes from 10 to 10_6 sporozoites or merozoites, or a mixture thereof, for each species that is included in the dose.

Other preferred domesticated birds to be vaccinated in accord with the method of the invention are game birds, ducks and ratites.

The method of the invention further comprises, in combination with the present vaccination method, administering in ovo an immune stimulant at any time during incubation.

A preferred method of administering the immune stimulant is simultaneously with the in ovo administration of a dose of sporozoites or merozoites, or mixture of said sporozoites and merozoites, during the final quarter of incubation.

DETAILED DESCRIPTION OF THE INVENTION

The present vaccination method involves the in ovo administration, during the final quarter of incubation, of live Eimeria sporozoites or merozoites, or a mixture thereof, into domesticated birds' eggs. In the case of chickens, in ovo administration is preferably done on days 15–20 of incubation, and most preferably on day 18 of incubation. In the case of turkeys, in ovo administration is preferably done on days 21–26 of incubation.

The present vaccination method can be performed using any suitable in ovo administration method. Preferably, the present vaccine is administered via injection. According to one method of injection, a hole is made in the egg shell at the large end 30 of the egg using an 18 guage needle to expose the egg's air cell. A 1.0–1.5 inch 22 guage needle attached to a syringe of appropriate size (1–3 ml) can be inserted through the hole and through the membrane of the air cell. An appropriate number of sporozoites or merozoites, or, when mixed, an appropriate number of sporozoites and merozoites, are suspended in a suitable liquid carrier, for instance 10–500 μl of phospbate-buffered saline, and then injected into the egg. The appropriate volume will depend on the size of the egg being treated, with ostrich eggs obviously being capable of taking more volume tban chicken eggs. The site of injection can be within any region of the egg or embryo. Preferably, injection is done axially through the center of the large end of the egg into the anion.

Alternatively, an automated egg injection system can be used in the present vaccination method. Such systems are described in U.S. Pat. Nos. 4,681,063, 4,040,388, 4,469,047, and 4,593,646, which are herein incorporated by reference. Other appropriate methods of injection are known to tbose skilled in the art.

Oocysts can be prepared by any of several methods known to those skilled in the art. Such methods include those described in J. F. Ryley et al., Parasitology 73:311–326, 1976 and P. L. Long et al., Folia Veterinaria Latina VII3, 201–217, 1976, wbicb are berein incorporated by reference. According to one method, commercial broiler chickens, approximately 2 weeks old, are infected with the Eimera species of interest by oral gavage of an appropriate dose of sporulated oocysts. For example, a typical dose used for *E. tenella* is 200,000 sporulated oocysts/bird. Well known procedures for collection and purification of oocysts from infected birds are then followed. For most species of Eimeria, feces are collected from infected birds 5–7 days post-infection, blended and filtered to remove debris, then centrifuged at a speed sufficient to pellet the remaining fecal material. For *E. tenella,* a similar procedure is used except that cecal cores are taken at 6 days post-infection. The pellet is resuspended in a saturated salt solution, in which the oocysts float and most of the contaminating debris can be removed by centrifugation. The oocyst suspension is then diluted to lower the salt concentration. The oocysts are washed repeatedly to remove the salt and resuspended in potassium dichromate Solution (2.5% w/v). The oocyst suspension is incubated at 29° C. with shaking (e.g., 140 rpm) for approximately 72 hours to induce sporulation of the oocysts. Alternatively, the oocysts can be treated with sodium hypochlorite and then sporulated. The number of sporulated oocysts/ml is determined by direct count using a hemocytometer, and the culture is stored, preferably under refrigeration until needed.

To prepare sporocysts, the potassium dichromate is removed from the oocyst suspension described above by repeated washing of the oocysts, which involves collection of oocysts by centrifugation and resuspending in deionized or distilled water. When the dichromate has been removed as judged by the lack of yellowish-orange coloration, the oocyst suspension is mixed with an equal volume of sodium hypochlorits (bleach) and incubated at room temperature for 15 minutes. The bleach is then removed by repeated washings, and the oocysts are resuspended in physiological saline or dejonized water. Oocysts can be broken to release sporocysts using a variety of known techniques. For example, oocysts can be broken to release sporocysts by mixing the oocysts with glass beads of 1–4 mm diameter and shaking by hand, vortex mixer, or shaking incubator, or using a hand-held homogenizer. Unbroken oocysts and oocysts walls can be separated from the released sporocysts by differential centrifugation in 50% PERCOLL., a colloidal suspension of polyvinyl pyrrolidone coated silica particles (sold by Pharmacia Biotech) or 1 M sucrose as described in Dulski et al., Avian Diseases, 32: 235–239, 1988.

To prepare sporozoites or a sporozoite-rich preparation to be used in accord with the present invention sporozoites are excysted from the sporocyst preparation described above. In one procedure, sporocysts prepared as described above are pelleted by centrifugation, resuspended in excystation buffer (0.5% taurodeoxycbolic acid and 0.25% trypsin in phosphate buffered saline, pH 8.0) and incubated with shaking for one hour at 41° C. A sample of the resulting suspension is counted to determine the number of sporozoites, the sporozoites are washed once to remove tbe excystation buffer, and resuspended in pbospbate-buffered saline at the desired concentration for in ovo injection. This preparation contains sporozoites, sporocysts and oocysts, and, without further purification, can be used in accord with the present vaccination method. Purified sporozoites, removed from sporocysts and oocysts, can be prepared by DE-52 anion

exchange chromatography as described in D. M. Schmatz et al., J. Protozool. 31: 181–183, 1984, which is herein incorporated by reference. The dose of sporozoites to be used in the present vaccination method will vary according to the type of domesticated bird being vaccinated and the species of Eimeria being used in the vaccine. In general, the dose can range from 10 to 10^5 sporozoites per egg. Preferably, the dose ranges from 10 to 10^5 sporozoites per egg, and, more preferably, the dose ranges from 10^2 to 10^5 sporozoites per egg.

Merozoites can be prepared by various methods known to those skilled in the art. In one method, sporozoites are infected into primary chick kidney cells (PCK) that are grown in culture as cell aggregates, using a modification of the method described in D. J. Doran, J. Parasit. 57: 891–900, 1971, which is herein incorporated by reference. PCK cells are grown at 40° C. in 3% CO_2 in modified RK2 medium-DMEM/F12 with L-glutamine and 15 mM HEPES, supplemented with fetal bovine serum, penicillin-streptomycin, 15 mM sodium bicarbonate, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenious acid and 0.01 μM hydrocortisone HCL as described in S. D. Chung et al., J. Cell Biol. 95: 118–126, 1982. PCK cells are prepared from kidneys of 2 to 3 week old chicks by mincing the kidney, and then treating the tissue at 37° C. with several changes of 0.2 mg/ml collagenase (sold by Worthington Biochemical Corp., Freehold, N.J.) in phosphate-buffered saline solution. The cellular aggregates in the supernatant are washed, resuspended in modified RK2 medium containing 5% fetal bovine serum and used to seed tissue cultures flasks at a density of 10^5 aggregates per cm^2. The PCK cells are incubated for 18 hours at 40° C. in 3% CO_2 and then infected with 4×10^5 sporozoites/cm^2. Infected cultures are grown in modified RK2 medium containing 2% fetal bovine serum. After 24 hours of incubation, to allow invasion, uninvaded sporozoites are removed by agitating the flask and removing the culture medium. The cell layer is washed once with modified RK2 medium containing 2% fetal bovine serum and the culture medium is again discarded. Fresh RK2 medium is added, and the cultures are incubated for another 48 to 54 hours until merozoites are released into the culture medium.

Purification of the merozoites to remove host cell debris can be done by various methods known to those skilled in the art. In accord with one method, as described in J. A. Olson, Antimicrob. Agents Chemother. 34: 1435–1439, 1990, the culture medium containing the released merozoites is collected and spun at 450×g for 10 minutes to concentrate the merozoites. The pellet containing the merozoites and the host cell debris is suspended in 0.1 M NaCl-0.05 M KCl-20% bovine serum albumin and applied to a DE-52 anion exchange column equilibrated in 75 mM Tris-40 mM NaH_2PO_4-86 mM NaCl-100 mM glucose at pH 8.2. Merozoites flow through the column. Merozoites collected from the column can be tested for purity by electron microscopy as described in A. Kilejian, J. Biol. Chem. 249: 4650–4655, 1974. In general, the merozoite dose can range from 10 to 10_6 merozoites per egg. Preferably, the dose ranges from 10 to 10^5 merozoites per egg, and, more preferably, the dose ranges from 10^2 to 10^5 merozoites per egg. When sporozoites and merozoites are mixed, in general, the dose comprising the total number of merozoites and sporozoites can range from 10 to 10^6 per egg. Preferably, the dose ranges from 10 to 10^5 merozoites and sporozoites per egg, and, more preferably, the dose ranges from 10^2 to 10^5 merozoites and sporozoites per egg.

The sporozoites or merozoites, or mixture thereof, can be injected in ovo in any physiologically suitable medium. Preferably, they are suspended in physiologically balanced saline such as phosphate-buffered saline. The selected medium can optionally include one or more suspending agents including physiologically suitable gels, gelatins, hydrosols, cellulose, or polysaccharide gums.

Preferably, in the present vaccination method, sporozoites or merozoites, or a mixture thereof, of two or more Eimeria species are injected in ovo at the same time. In accord with the present vaccination method, sporozoites or merozoites, or a mixture thereof, of all identified species of Eimeria that infect a specific domesticated bird, such as chicken, can be injected in ovo at the same time, or in series, at appropriate doses to provide immunological protection against all species.

Immune stimulants can be used in conjunction with the present vaccination method. Suitable immune stimulants include, but are not limited to, cytokines, growth factors, chemokines, supernatants from cell cultures of lymphocytes, monocytes, or cells from lymphoid organs, cell preparations or cell extracts (e.g. *Staphylococcus aureus* or lipopolysaccharide preparations), mitogens, or adjuvants including low molecular weight pharmaceuticals. An immune stimulant can be administered in ovo at any time during incubation. Preferably, an immune stimulant is administered in ovo in the medium containing the dose of Eimeria sporozoites or merozoites, or mixture thereof.

The efficacy of the present invention in vaccinating against coccidiosis is illustrated in the following examples. Each dose was injected in ovo in physiologically-acceptable saline as described above. The effectiveness of a particular preparation was determined by monitoring its effect on hatch rate and hatch weight of the chicks, and, following challenge infection, oocyst production, weight gain, and pathogenicity (lesion score). Lesion scores were assigned according to the protocol described in J. K. Johnson and W. M. Reid, Exp. Parasitol. 28: 30–36, 1970. according to which a value of 0 represents no disease and a value of 4 represents maximum pathology.

EXAMPLE 1

Chicken eggs were injected on day 18 of incubation with a preparation containing 10^5 *E. tenella* sporozoites per egg. The preparation was not purified to remove sporocysts and oocysts. Each dose also contained approximately 10^4 *E. tenella* sporocysts and approximately 10^4 *E. tenella* oocysts. As a control, eggs were injected only with phosphate-buffered saline solution. In the sporozoite-treated population of birds the mean oocyst shed at 7 days post-hatch was 1.1×10^6 oocysts/bird. Non-immunized birds and the sporozoite-treated birds were challenged with various doses of sporulated oocysts of *E. tenella*, administered by oral gavage, on days 7, 14 or 21 post-hatch. The data appear in Table 1.

TABLE 1

Immunized Versus Non-Immunized Birds: Responses To Different Challenge Doses At Different Times Post-Hatch

Group[1]	Challenge dose (sporulated oocysts per bird)	Lesion score on sixth day after challenge: Non-immunized	Lesion score on sixth day after challenge: Immunized In Ovo (10^5 sporozoites on day 18 of incubation)	Weight gain (grams) per bird over six day period after challenge: Non-immunized	Weight gain (grams) per bird over six day period after challenge: Immunized In Ovo (10^5 sporozoites on day 18 of incubation)
Challenge on day 7 post-hatch	0	0	0	188	167
	2.5×10^2	3.0	0.3*	154	153
	5×10^3	3.1	1.2*	153	167
	1×10^4	3.7	1.4*	123	165*
Challenge on day 14 post-hatch	0	0	0	266	278
	2.5×10^3	2.4	0.4*	260	269
	5×10^3	2.8	1.1*	244	265
	1×10^4	3.3	1.6*	235	259
Challenge on day 21 post-hatch	0	0.1	0	361	357
	2.5×10^3	2.3	0.7*	375	358
	5×10^3	2.1	0.8*	332	344
	1×10^4	2.8	1.4*	337	395*

[1]Each group was subjected to a single challenge of sporulated oocysts on days 7, 14, or 21 post-hatch. For example, birds challenged on day 21 post-hatch were not challenged on days 7 or 14 post-hatch.
*Significantly different from non-immunized birds ($p < 0.05$, ANOVA)

The data in Table 1 clearly show that the immunized birds were less susceptible infection than their non-immunized hatch mates as indicated by the reduced lesion scores and improved weight gains in the immunized birds. The data also demonstrate that the method of the invention imparts immunity to the chicks at a relatively early age (within seven days post-hatch). Furthermore, the data show that the immunity continues as the chicks grow and develop. Imparting immunity to chicks at an early age provides a significant advantage in the broiler chicken industry because broilers routinely reach market by 6 weeks of age.

EXAMPLE 2

Chicken eggs were injected on day 18 of incubation with saline (control) or preparations containing different doses of sporozoites of *E. tenella*, as indicated in Table 2 which provides the pre-challenge results. The sporozoite preparation used for each dose contained 62% sporozoites, 9% sporocysts, and 29% oocysts. Each dose containing 10^5 sporozoites included a total of 1.6×10^5 parasite stages porocysts, and oocysts).

Effect of in ovo Vaccination on Hatch Rate and Weight

TABLE 2

Sporozoites injected per egg on day 18 of incubation	Hatch rate (%)	Hatch weight (grams)
0	94	48.2
0	94	48.0
10^3	100	49.4
10^4	97	47.0
10^5	94	49.1

The data in Table 2 show that chicks hatched from eggs injected with live sporozoites were substantially identical to their non-immunized hatch mates in terms of hatch weight. The chicks were then challenged on day 14 post-hatch hatch with 1.25×10^4 sporulated oocysts of *E. tenella* per bird, administered by oral gavage. The post-challenge results are provided in Table 3.

Response to Challenge Infection of Non-immunized Birds Versus Birds Treated in ovo with Different Doses of Sporozoites

TABLE 3

Sporozoites injected per egg on day 18 of incubation	Weight Gain per bird over six day period after challenge	Lesion Score on day 6 after challenge	Oocysts shed per bird ($\times 10^6$) on day 6 after challenge
0	278	3.2	12.3
0 (Unchallenged control)	321	0	0.003*
10^3	289	2.6*	11.2
10^4	291	2.7	12.2
10^5	304*	1.4*	1.4*

*Significantly different from non-immunized group (received saline) that was subjected to challenge ($p < 0.05$, ANOVA)

The data in Table 3 show that for every parameter (weight gain, lesion score, oocyst shed) chicks hatched from eggs injected with different doses of the sporozoite preparation showed evidence of immunity. In comparison to the control birds that were treated only with saline and were subjected to challenge infection, the birds immunized in ovo with the sporozoite preparation showed greater weight gain and reduced lesion scores. In addition, the birds immunized in ovo with the sporozoite preparation passed fewer oocysts than the control birds after challenge infection, indicating that the infection was less severe in the immunized birds.

We claim:

1. A method of vaccinating a domesticated bird against coccidiosis comprising administering in ovo, during the final quarter of incubation, an effective immunizing dose of live Eimeria sporozoites or merozoites, or a mixture thereof.

2. The method of claim **1** wherein the dose comprises 10 to 10^6 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10 to 10^6.

3. The method of claim **1** wherein the dose comprises 10^3 to 10^6 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10^3 to 10^6.

4. The method of claim **1** wherein the dose comprises 10^2 to 10^5 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10^2 to 10^5.

5. The method of claim **2** wherein the domesticated bird is a chicken.

6. The method of claim **5** wherein the dose comprises sporozoites or merozoites, or a mixture thereof, of two or more species of Eimeria selected from the group consisting of *E. tenella, E. acervulina, E. maxima, E, necatrix, E. mitis, E. praecox,* and *E. brunetti.*

7. The method of claim **6** wherein the dose is administered by in ovo injection.

8. The method of claim **2** and further comprising administering in ovo an immune stimulant at any time during incubation.

9. The method of claim **8** wherein the immune stimulant is administered in ovo simultaneously with the dose of sporozoites or merozoites, or mixture of said sporozoites and merozoites.

10. The method of claim **2** wherein the dose comprises merozoites.

11. The method of claim **2** wherein the dose comprises sporozoites.

12. The method of claim **11** wherein the sporozoites have been purified to remove sporocysts and oocysts.

13. The method of claim **2** wherein the domesticated hird is a turkey.

14. The method of claim **13** wherein the dose comprises sporozoites or merozoites, or a mixture thereof, of two or more species of Eimeria selected from the group consisting of *E. meleagrimitis, E. adenoeides, E. gallopavonis, E. dispersa, E. meleagridis, E. innocua,* and *E. subrotunda.*

15. The method of claim **14** wherein the dose is administered by in ovo injection.

16. The method of claim **2** wherein the domesticated bird is selected from the group consisting of: a game bird, duck and ratite.

* * * * *

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Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/749,602	EMERY ET AL.	
	Examiner	Art Unit	
	Patricia Leith	1655	

– The MAILING DATE of this communication appears on the cover sheet with the correspondence address –

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *5/7/09*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *34-44, 67-69, 71-82, and 84 –102* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *34-44, 67-69, 71-82, and 84 –102* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *5/7/09*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

. DETAILED ACTION

Claims 34-44, 67-69, 71-82, and 84 –102 remain pending in the application and were examined on their merits. There were no claim amendments submitted by Applicants in the interim between the previous non-final Office action and this final Office action.

Rejections Removed

The following rejection has been removed due to Applicants' persuasive arguments that the combination of references does not make obvious the claimed invention:

Claims 34-44, 67-69, 71-82, and 84 –102 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Emery et al. (US 5,830,479) in view of Phelps et al. (US 5, 339,766) and further in view of Evans et al. (US 6,500,438 B2) in view of Genovese et al. (1998) in light of Sharma et al. (US 4458630 A)*.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 34, 37, 39-43, 67-69, 83-86, 89, 91-95 and 97-102 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Emery et al. (US 5,538,733) in view of Emery et al. (US 5,830,479) in view of Phelps et al. (US 5, 339,766).

Emery et al. (US 5,538,733) discussed the problem of vaccination of young animals in that maternal antibodies present in neonates may interfere with an animal's immune response, while proposing a solution of administration of vaccines

present in sustained and delayed delivery agents to young poultry between the ages of 1-90 days (see entire reference and Abstract). Emery et al. explicitly indicated that the method advantageously incorporated injection of an 'implant matrix' made of "...biocompatible, biodegradable, bioabsorbable and/or bioerodible polymeric material.." such as cholesterol and cellulosic polymers to "...release the immunogen for sustained delivery into surrounding tissue fluids over an about 1-90 day period" (see, col. 2, lines 15-55).

Emery et al. specifically indicated that "The continuous presence of a priming dose of the immunogen provides and effective way of priming a young animal so that a secondary immune response to a pathogenic infection is stimulated substantially immediately when passive protection by maternal antibodies *against the pathogen* is no longer effective" (see paragraph bridging columns 3-4, emphasis added). Hence, it is clear that Emery et al. is stating that the immunized poultry possess the maternal antibodies against the same immunogen used to inoculate the animals including domestic fowl (see also col. 9, lines 41-49).

Emery et al. indicated that the 'time-delayed implant' "...will substantially maintain integrity of the matrix for *a desired length of time*. Preferably, the matrix will remain intact for up to about 3 weeks, or after the level of maternal antibody has significantly declined, at which time the antigen is released from the matrix."

(emphasis added) Hence, the matrix is formulated for delayed delivery. Emery et al. further indicate that the matrix is formulated for delayed and sustained delivery: "The matrix may optionally be formulated to include a soluble or insoluble pore-forming agent that will dissipate from the matrix into surrounding tissue fluids causing the formation of pores and/or channels throughout the implant matrix....sodium chloride...carboxymethylcellulose" (see col. 9, lines 29-39).

A preferred immunogen for implantation disclosed by Emery et al. was siderophore receptor protein (SRP) from gram negative bacteria (see col. 7, line 50-col. 8, line 24). See also Example 2, wherein a sustained/delayed release formulation of SRP is administered to 1 day old turkey poults to establish immunity *against the SRP* indicative of an adaptive immune response. Notably, in this Example, Emery et al. specifically indicate that a further preference for delivery time to is between 1-60 days of age.

Emery et al. additionally taught the advantageous nature of administration of a booster "...to stimulate a secondary immune response in the animal," wherein the booster was an SRP. Emery et al. gave a specific example wherein a 21 day implant administered to a turkey poult is given a booster injection after the expiration of the implant, stated by Emery et al. to be 21 days after implantation, at about 28-48 days to stimulate the immune response (see col. 11, lines 1-19). This booster time

disclosed by Emery et al. falls completely within the claimed booster time of 3-12 weeks (equates to 21-84 days). Serological profiles to quantify antibody titers to SRP were known at the time of Emery et al. and specifically discussed (see col. 11, lines 33-58).

Emery et al. did not specifically teach wherein the siderophore receptor was administered *in-ovo* at 'a time when maternal antibodies of the bird to the immunogen are sufficiently reduced'. Nor did Emery et al. teach the specific injection times as found in claims 39-42 and 44 or wherein a second dose of immunogen was given at 3-12 weeks post-hatching (claim 43). Emery et al. further did not teach the incorporation of porins into their vaccine.

Emery et al. (US 5,830,479) disclosed a method for immunizing poultry with a siderophore from gram-negative bacteria wherein the siderophore is enterochelin or siderophore citrate as examples (col.s 1-53, particularly col. 5, lines 29-38 and claims 1 and 3). As stated by Emery et al. "The vaccine of the present invention may be used for preventing and eliminating infections of gram-negative bacteria in poultry and other animals including humans" (col. 11, lines 9-12). Emery et al. specifically suggested sustained release administration of the vaccine (col. 11, line 15) and *in-ovo* administration in poultry: "The vaccine of the present invention may be used for

preventing and eliminating infections of gram-negative bacteria in poultry and other animals, including humans....may be delivered to the animal, for example, by...egg inoculation (i.e., poultry...by known techniques in the art...the vaccine contains an amount of a siderophore receptor protein to stimulate a level of active immunity in the animal to inhibit and/or eliminate gram-negative bacterial pathogenesis and/or sepsis" (col. 11, lines 10-21). Emery et al. specifically taught that "The protein may also be incorporated into a carrier which is a [*sic*] biocompatible and can incorporate the protein and provide for its controlled release or delivery, for example, a sustained release polymer such as a hydrogel, acrylate, polylactide, polycaprolactone, polyglycolide or copolymer thereof...an example of a solid matrix for implantation into the animal and sustained release of the protein antigen into the body is a matabolizable matrix, as described...in US ...4,452,775 (Kent)" (col. 11, lines 27-36). Emery et al. also taught the advantageous use of a booster vaccine given "21-28 days after the first injection" and the use of adjuvants such as porins from gram negative bacteria for administration along with SRP's (see, col. 7, line 50-col. 8, line 9). Emery et al. offered that the amount of vaccine was varied in order to achieve optimal vaccination (see col. 11, line 49- col. 12, line 6).

Phelps et al. (US 5, 339,766) disclosed a method for introducing mat6erinl into poultry eggs during early embryonic development which included injection of a therapeutic substance contained within a biodegradable matrix such as polylactide

polymers (lactides/glycolides) directly into the developing bird egg. Materials intended for delivery included "vaccines, vitamins, antibiotics hormones, enzyme inhibitors, peptides, cells, DNA and other therapeutic molecules" (col. 3, lines 33-36). Phelps et al. discussed that, "Eggs treated by the method of the present invention are preferably fertile eggs which may be in any period of incubation, from early to late... " (col. 4, lines 15-18). Phelps et al. further explained that "Such beneficial effects included increased growth, disease resistance due to in ovo vaccination, increased percentage hatch of multiple incubated eggs, and otherwise improved physical characteristics of hatched poultry" (col. 1, lines 20-24).

One of ordinary skill in the art would have been motivated to administer a sustained-release formulation *in ovo*, to a bird (i.e., poultry such as chicken) wherein the formulation comprised a siderophore receptor such as enterochelin, and wherein the sustained-release formulation was sustained until the hatching of the bird (i.e., 1-60 or 1-90 days post-hatching) in order to increase the bird's immune system to foreign disease causing bacteria. It was clear from the prior art that siderophore receptors from gram-negative bacteria were known to vaccinate birds, and suggested for use *in-ovo* by Emery et al. '479. Further disclosed by Emery et al. as well as Phelps et al. were suitable mediums and sustained release biocompatible matrices for *in-ovo* injection of vaccines. The ordinary artisan would have recognized, in view of Emery '773 that sustained release of SRPs to young poultry or poultry embryos (in-ovo) would need to be formulated to release the SRPs at a time that the immunogen is "sufficiently reduced

so that the birds are capable of mounting an adaptive immune response". This knowledge in the art of poultry immunization is made perfectly clear by Emery '773 and is not considered a novel idea. It is clear from the teachings of the references as a whole, that the Emery et al. patent '773 although not teaching egg inoculation of their sustained/delayed release matrix SRP vaccine is cured by the subsequent Emery '479 patent which clearly suggests that the same matrix, including SRP and advantageously the addition of porin as an adjuvant, into an egg to vaccinate young poultry.

In-ovo vaccination techniques as claimed were known and well-utilized and rendered obvious at the time the Invention was made as evidenced by Phelps et al. Emery et al. '773 and Emery et al. '749 together taught optimal times for vaccinating young poultry at a time when maternal antibodies were reduced in order for the bird to mount an immune response; Emery et al. '773 teaching specifically times advantageous to administer such a vaccine which included SRP when maternal antibodies to SRP were 'sufficiently reduced': the 'time-delayed implant' "...will substantially maintain integrity of the matrix for *a desired length of time*. Preferably, the matrix will remain intact for up to about 3 weeks, or after the level of maternal antibody has significantly declined, at which time the antigen is released from the matrix." It is the opinion of the Examiner that at the time the invention was made, the claimed invention was well-within the purview of the ordinary artisan. Time delayed/sustained matrices for delivering SRP to poultry were known in the art at the time the invention was made and known to be manipulated to release SRP at a desired time. Clearly, the results

achieved by both Emery et al. patents include successful vaccination of young poults anywhere from injection at one day (with the sustained delivery matrix of Emery et al. '773), three weeks (see Example 3, Emery et al. '479), six weeks (Emery et al. '479).

Clearly, there is no explicit time indicated in the prior art nor the Instant specification of 'until the maternal antibodies in a bird hatching from the egg are reduced so that the bird is capable of mounting an adaptive immune response to the immunogen' because this time would vary from bird to bird. Hence the reason for the delayed/sustained release formulations of both Emery et al. patents. Such a formulation intended for sustained/delayed release would provide continual vaccine delivery over a desired amount of time in order to successfully vaccinate young birds.

Claims 34-44, 67-69, 71-82, and 84 –102 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Emery et al. (US 5,830,479) in view of Phelps et al. (US 5, 339,766) in view of Emery et al. (US 5,538,733).and further in view of Evans et al. (US 6,500,438 B2).

The teachings of Emery et al. '749, Emery et al. '773 and Phelps '776, were discussed *supra*. None of these references specifically taught t the specific injection protocols as recited in claims 35, 36, 38 and 44.

Evans et al. (US 6,500,438 B2) taught a method for *in ovo* vaccination of chickens with *E. sporozoites* via injection, wherein the injection was preferentially performed in the final quarter of incubation or specifically at day 18 of incubation, however would have been effective during any time of incubation (col. 2, lines 1-6, col. 3 lines 25-27 and Example 1).

Hence, although the prior art did not teach a specific embodiment where SRP was injected into bird eggs at the claimed injection times as required by claims 35, 36, 38 and 44, it has been held that where the general conditions of a claim are disclosed in the prior art, discovering the optimum or workable ranges involves only routine skill in the art. *In re Aller*, 220 F2d 454,456,105 USPQ 233; 235 (CCPA 1955). see MPEP § 2144.05 part II A. It is clear from the prior art teachings as a whole that the sustained release matrices including SRP and advantageously including porins as an adjuvant to the SRP vaccine were formulated to release during a time that maternal antibodies to the vaccine were sufficiently reduced in order for the chick to produce antibodies to the vaccine. Such matrices were well-known in the art and producing such compositions was within the skill level of the ordinary artisan at the time the invention was made. In-ovo injections to produce an immune response were further known in the prior art to be carried out within the time frames specified by the claims. There is no one limitation within the claims which is deemed to be directed toward a novel invention; as the prior art provides a clear roadmap to the claimed invention.

The Supreme court has acknowledged:

> When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. **If a person of ordinary skill can implement a predictable varition..103 likely bars its patentability**...if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond that person's skill. A court must ask whether the improvement is more than the predictable use of prior-art elements according to their established functions...
>
> ...**the combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results** (see *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385 U.S. 2007) emphasis added.

From the teachings of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Response to Arguments

Applicants' arguments concerning these remaining rejections as set forth *supra* were very carefully considered; however, were not found to be convincing to obviate said rejections.

Applicants first argue:

> Immune tolerance to a foreign antigen can occur when a subject is exposed to a foreign antigen under conditions that elicit specific unresponsiveness to the foreign antigen rather than an adaptive humoral immune response to the antigen. In other words, under some circumstances, exposure to a foreign antigen does not necessarily result in the challenged subject mounting an adaptive immune response, but instead results in the subject's immune system perceiving the foreign antigen as "self" and establishing antigen-specific immune non-response.
> Many of the conditions under which immune tolerance may be induced are present in the circumstance of in ovo vaccination as recited in Applicants' claims. (See, Microbiology, fourth edition, Davis et al. eds., 1990, J.B. Lippincott Co., Philadelphia, Pennsylvania, pp. 381-382.) For example, SRPs-the immunogens specifically identified in the Office Action-are monomeric antigens, not aggregated; sustained release implants are not equivalent to injection into tissue, but are more similar to intravenous administration; and in ovo administration necessarily results in vaccination of the embryo rather than adult. So, one skilled in the art would recognize that vaccinating embryos or newly-hatched chicks using sustained release implants harbor the risk of inducing immune tolerance to the immunogen in the vaccine rather than raising adaptive immunity against the immunogen. Many of these conditions are present whether the sustained release implant is administered at one day of age (as in the '733 patent) or in ovo, as in the present claims (pp. 7-8 Remarks).

However, Applicants' arguments regarding the fact that an immune response to the antigen may not have occurred due to *in-ovo* vaccination of chicks using sustained release would be an exception to the rule and is not a persuasive argument. The skilled artisan would have understood at the time the invention was made that vaccinations are not 100% successful. Based upon the combined teachings of the prior art, the ordinary artisan would have had a reasonable expectation of success; the prior art already recognized the use of SRPs for vaccination into poults as well as in-ovo via use of sustained delivery matricies. It is evident upon reading the references in combination; that the ordinary artisan, considering the successfulness of vaccination using SRP proteins in young chicks, that delivery of SRP's comprising a delayed release matrix, which was already taught by the prior art, to release at times already known to be

successful for vaccinating young birds would have been prima facie obvious at the time the invention was made. There is no indication in the Specification that Applicants' have achieved any unexpected result over what was already clearly suggested by the combination of the prior art teachings and hence, absent such an unexpected result, the claimed invention is deemed an obvious combination of prior art elements.

Applicants argue:

> The difference between vaccinating an egg by sustained release of a selected immunogen from a biocompatible implant at one day of age versus injecting the biocompatible implant in ovo-and a compelling reason why one skilled in the art would not extend the teaching of the '733 patent to in ovo delivery-is that *the risk of inducing immune .tolerance to the immunogen is greater when the biocompatible implant containing the immunogen is delivered in ovo compared to delivering the biocompatible implant after hatch. One reason for the increased risk of inducing immune tolerance when the biocompatible implant is injected in ovo is the different amounts of-and the corresponding effects of the different amounts of-circulating maternal antibody in the embryo versus in the newly-hatched chick.*
> Each of claims 34, 69, and 84 recites that the egg (or eggs) into which the biocompatible implant is injected comprises maternal antibody to the selected immunogen. Prior to hatch, some of the maternal antibody circulates in the embryo but most remains sequestered in the yolk. At hatching, however, the yolk is fully absorbed and the maternal antibody from the yolk is fully absorbed into the circulation of the chick. Thus, the chick-but not the embryo-has the full passive immunization benefit of the maternal antibodies. Consequently, the circulating maternal antibody environment is very different in the embryo than in the newly-hatched chick and this difference influences the risk of inducing immune tolerance. (p. 8, Remarks, emphasis added).

However, Applicants' assertions are unsubstantiated and found unpersuasive in view of the prior art teachings. Emery '733 explicitly taught *in-ovo* vaccination using sustained/delayed release of SRP antigen to vaccinate poultry. Although there is no specific example of where Emery performed *in-ovo*

vaccination; there is an explicit suggestion for the ordinary artisan to perform *in-ovo* vaccination by Emery et al. and further, *in-ovo* vaccination techniques were well-known and conventional in the art at the time the Invention was made. While Applicants point to unknown parameters and unpredictability with regard to *in-ovo* vaccination, the level of unpredictability in the Instant case does not rise to the level of patentability considering that the prior art explicitly suggested *in-ovo* vaccination of birds, specifically taught the delayed/sustained release times of the claims and specifically taught the use of SRP proteins for these purposes. The only teaching lacking between the two Emery et al. patents and the claimed invention is the age of the egg at vaccination. However, vaccination at this time was known in the art as disclosed by Evans et al. It is deemed, in light of the combination of prior art references; that the ordinary artisan, having the knowledge that SRP proteins were advantageously administered to young poultry to vaccinate them against harmful bacteria, that SRP proteins were advantageously formulated to be delivered by sustained/delayed release until a time when 'maternal antibodies...are reduced so that the bird is capable of mounting an adaptive immune response,' that SRP proteins were specifically suggested to be delivered *in-ovo* with sustained release, that the claimed times for release of the SRP were known in the art and that *in-ovo* vaccination techniques were also well-known in the art (Phelps et al.), and vaccination at the times as indicated by the claims were also known in the art (Evans et al.).

Thus, absent any evidence of an unexpected result, the claimed invention is deemed an obvious method already suggested by the prior art teachings. The ordinary artisan, relying on the above-cited US Patents would have had a reasonable expectation of success in producing the claimed method. Although neither Emery et al. patents explicitly demonstrated *in-ovo* vaccination of SRP proteins at the age of the egg as indicated by claims 35 and 36 for example, determining a time to vaccinate poultry eggs with known vaccines such as SRPs which were already known to be delivered in delayed/sustained release matricies at the times as required by the claims is deemed well-within the skill level of the ordinary artisan and would have been achieved through routine optimization/experimentation. The Specification as a whole appears to be solving an asserted problem of delivering an SRP protein to a young poult in such a manner as to deliver said SRP at a time when maternal antibodies are reduced. However, it appears that the problems asserted by the Specification *were already solved by the teachings of the prior art US Patents*. There is nowhere in the Specification which suggests that the time of vaccination is particularly crucial or where these times were found to achieve a superior result. The prior art as well as the claims teach a broad window for sustained/delayed release of the SRP antigen to deliver to a poultry; this is because it appears that there is no absolute time which is known when maternal antibodies will be at the lowest level to ensure maximum protection since this time would vary from bird to bird. Absent such additional information which would demonstrate that

Applicants have achieved a result which was not already found predictable based upon the combination of the prior art teachings; it is decidedly taken that the vaccination times were an obvious choice based upon the consideration that eggs were routinely vaccinated at these times. Hence, Applicants' arguments as presented on page 9 of their remarks concerning day old chicks and the amount of maternal antibody are unpersuasive. Applicants are claiming a method which was already suggested by the prior art; the maternal antibody titers of the chicks were not measured in Applicants data present in the Specification; it is unknown exactly when the maternal antibodies to an SRP protein will be sufficiently low to amount a maximum immune response; however, the prior art already recognized that SRPs could be delivered via sustained/delayed matricies in order to release the SRP proteins within a broad window to achieve a desired immune result (i.e., vaccination). It does not appear that Applicants have gone above-and-beyond what was already known and expected from SRP vaccination of poultry as disclosed by the prior art.

Applicants' arguments regarding that when a biocompatible implant is provided *in ovo*, the level of maternal antibody absorbed by the chick...at day 20 of incubation...is incomplete, and as a consequence, the embryo is at risk for developing immune tolerance (p. 9, Remarks) is not found convincing. The prior art already recognized that the presence of maternal antibodies in young poultry hastened the need for delivery of vaccines at a time when maternal antibodies in

the poultry were reduced (both Emery et al. US Patents). Additionally, these statements are not provided in the Specification, and there is no evidence given in the Specification that the time chosen for vaccinating the eggs was significant. Absent such indication; seeing that the prior art already specifically suggested *in-ovo* vaccination with SRP proteins using biocompatible implants which provide for sustained/delayed release for the same delivery times as required by the claims, Applicants' age of egg to be vaccinated as claimed is deemed obvious since these were well-known times to vaccinate chicks *in-ovo*. Considering that the prior art already taught that the biocompatible matrix could have been formulated to release implant "....for *a desired length of time*. Preferably, the matrix will remain intact for up to about 3 weeks, or after the level of maternal antibody has significantly declined, at which time the antigen is released from the matrix" (Emery et al., 479, see citation above) the ordinary artisan would have had a reasonable expectation of success in carrying out the claimed method.

Applicants' arguments regarding the embryo's immune system and Applicants' statements that the effect of administering the sustained release implant to an embryo is unpredictable is not found convincing. While there is an acceptable amount of unpredictability regarding the immune functions of poultry embryos, if there were unpredictability in the art regarding this immunity, the unpredictability still exists in this application because there is no indication in the Instant specification that Applicants' have achieved any result which was not

already expected by the prior art. Applicants' only example regarding *in-ovo* inoculation merely quantitated live births and evaluated the injection sites. Applicants did not state exactly how the biocompatible matrix was prepared for this example (i.e., a determined release time), did not measure the maternal antibody titers of the eggs, did not measure antibody titers of the chicks after hatching and thus did not evaluate the overall success of a 20 day old egg vaccination. Nor did Applicants provide any comparative data which would demonstrate that an egg at day 20 of incubation would provide for any result which would not be expected by the prior art (i.e., vaccination). It appears that this example, Example 4 in the Specification; the only example in the Specification pertaining to *in-ovo* vaccination; was set forth to assess the toxic nature of the injection itself, as there is no subsequent data at all concerning these inoculated eggs. Hence, even considering if there is unpredictability in the art with regard to vaccination of poultry eggs 1) the prior art suggested *in-ovo* vaccination of SRP proteins using sustained/delayed release matricies, 2) the prior art taught the advantageous nature of delivering SRP proteins in biocompatible sustained/delayed release matricies to deliver SRP proteins to poults when decreased maternal antibodies were present and 3) *in-ovo* vaccinations were routine in the art and 4) *in ovo* vaccinations were successfully achieved by injecting antigens at the times recited by the claims. Hence, if the Examiner had reason to believe that the claimed composition was unpredictable, the claims themselves would be rejected under 35 USC 112 First paragraph,

because if a large degree of unpredictability existed, Applicants' specification would not cure this deficiency considering the lack of teachings in the Specification with regard to in-ovo inoculation. Nevertheless, it is taken from the prior art as a whole that the claims are enabled because the prior art already taught successful vaccination of young poultry with SRP proteins via use of a biocompatible matrix designed to provide sustained/delayed release until a time when maternal antibodies were sufficiently reduced so the bird could mount an immune response to the immunogen (SRPs) and because SRPs delivered in such matricies were specifically suggested for in-ovo administration. Considering the breadth of information concerning poultry vaccination with SRPs, the prior art is deemed enabling and the artisan would have had a reasonable expectation of success in carrying out the claimed invention.

Applicants' argue:

> As explained by Dr. Emery during the interview, the methods recited in claims 34, 69, and 84 permit those in the poultry industry to vaccinate a generation of eggs, at one time, and ensure that the resulting chicks can raise an adaptive immune response against a selected immunogen at the time-which can vary from egg to egg in a single generation from a single hen-when maternal antibody to the selected immunogen wanes. In the absence of such sustained release vaccination methods, those in the industry must otherwise vaccinated each chick every day over a multi-week period to ensure protection for the entire new generation because the waning of maternal antibody, if ever present at all, can vary from chick to chick. (p. 10, Remarks).

The Examiner does not disagree to the statement that "...claims 34, 69, and 84 permit those in the poultry industry to vaccinate a generation of eggs, at one

time, and ensure that the resulting chicks can raise an adaptive immune response against a selected immunogen at the time-which can vary from egg to egg in a single generation from a single hen-when maternal antibody to the selected immunogen wanes." However, the *combination of the prior art already taught this information* and the ordinary artisan, having the above-cited references before him or her would have had a reasonable expectation of success in carrying out the claimed invention based upon those teachings.

[If]... there are [a] finite number of identified, predictable solutions, [a] person of ordinary skill in art has good reason to pursue known options within his or her technical grasp, and if this leads to anticipated success, it is likely product of ordinary skill and common sense, not innovation *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385 U.S. 2007.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Patricia Leith whose telephone number is (571) 272-0968. The examiner can normally be reached on Monday - Friday 8:30am-5:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Terry McKelvey can be reached on (571) 272-0775. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Patricia Leith
Primary Examiner
Art Unit 1655

/Patricia Leith/
Primary Examiner, Art Unit 1655

PATENT
Docket No. 293.00010102

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Daryll A. EMERY et al.	Group Art Unit:	1655
Serial No.:	10/749,602	Examiner:	Patricia A. Leith
Confirmation No.:	8548		
Filed:	December 31, 2003		
For:	IN OVO DELIVERY OF AN IMMUNOGEN CONTAINING IMPLANT		

RESPONSE

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Office Action mailed January 7, 2009, please amend the above-identified application as follows:

Remarks begin on the page entitled "Remarks."

Remarks

The Office Action mailed January 7, 2009 has been received and reviewed. Claims 34-44, 67-69, 71-82, and 84-102 are pending and under consideration. Reconsideration and withdrawal of the rejections are respectfully requested.

Interview Summary

Applicants thank Examiner Leith for the courtesy of a telephonic interview held April 17, 2009. Inventors Daryll Emery, Ph.D. and Darren Straub, Ph.D., and Applicants' undersigned representative Christopher Gram participated on behalf of Applicants.

All of the pending claims and outstanding rejections were discussed, although the rejections based, at least in part, on Genovese *et al.* were discussed in particular. No agreement on claims was reached, but Examiner Leith agreed that Genovese *et al.* fails to provide teaching related to adaptive immunity.

The substance of Applicants' remarks during the interview are reflected in the remarks that follow.

The 35 U.S.C. §103 Rejection

Claims 34, 37, 39-43, 67-69, 83-86, 89, 91-95, and 97-102 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766) in view of Genovese *et al.* (1998) in light of Sharma *et al.* (U.S. Patent No. 4,458,630). Applicants respectfully traverse.

Claims 34-44, 67-69, 71-82, and 84-102 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766) and further in view of Evans *et al.* (U.S. Patent No. 6,500,438) in view of Genovese *et al.* (1998) in light of Sharma *et al.* (U.S. Patent No. 4,458,630). Applicants respectfully traverse.

Claims 34, 69, and 84 are independent. Each of the remaining claims depends, directly or indirectly, from one of the independent claims and, therefore, includes all of the features recited in the independent claim and any intervening claim from which it depends. Thus, remarks that refer to one or more of the independent claims apply equally to any claim that depends from an identified independent claim.

Applicants respectfully submit that the suggested combination of references fails to establish a *prima facie* case of obviousness for at least the reasons of record, which were more thoroughly discussed during the interview and are reiterated in the remarks that follow.

The Office Action asserts that Applicants' remarks regarding these rejections in the response filed October 10, 2008 were unpersuasive. In partial reply, the Office Action reiterates the assertion that Emery *et al.* would motivate one skilled in the art to innoculate successive generations *in ovo* using siderophore receptor antigens. The Office Action further asserts, "Inoculation of an egg with a siderophore receptor present in a chicken previously inoculated when it was an embryo with the same siderophore receptor would necessarily contain maternal antibodies to the siderophore receptor." (Office Action, page 6). Applicants interpret this statement to mean that the Office Action asserts that a hen inoculated *in ovo* against a siderophore receptor will necessarily pass maternal antibodies against the siderophore receptor to her own eggs. If Applicants' interpretation misconstrues the Office Action's position, Applicants' respectfully request clarification and that a subsequent Office Action be issued as non-final in order to give Applicants a fair opportunity to respond to the clarified position. The Office Action provides no technical support for the position that a hen inoculated *in ovo* against a particular antigen will pass maternal antibodies against that particular antigen to her eggs. Applicants respectfully submit that this premise is incorrect.

The fact that a hen has been immunized–whether *in ovo* or at any time during her life–against a particular antigen does not provide any information about the presence and/or content of maternal antibodies that she may pass to an egg. Humoral antibodies raised against an antigen do not persist in the individual indefinitely. Adaptive immune memory is provided by

the circulation of B cells that can recognize the antigen upon subsequent exposure to the antigen and thereafter generate a new and transient burst of humoral antibody against the antigen. The half-life of typical circulating humoral antibodies is on the order of days (e.g., 10 to 30 days). Thus, circulating humoral antibodies produced as a result of *in ovo* inoculation are catabolized long before an *in ovo*-inoculated chick can mature and lay eggs, even though the *in ovo*-inoculated bird remains protected against subsequent exposure to the antigen by circulating B cells. Consequently, while it may have been within the skill of the ordinary artisan to inoculate successive generations of eggs *in ovo* against siderophore receptor antigens, doing so does not teach or suggest that each successive generation of eggs will possess maternal antibodies against the siderophore receptor antigens.

The Office Action states that "the crux of the Genovese *et al.* publication is to vaccinate young poults against bacterial infection; namely, *Salmonella enteriditis*." (Office Action, page 7). This is incorrect.

Genovese *et al.* acknowledge the difficulty of vaccinating young poults because "the typical humoral/cell-mediated [i.e., adaptive] immune response requires 7 to 10 days to reach protective levels while poultry have been shown to be most susceptible to bacterial species such as Salmonella during the first 4 days of life. In addition, maternal antibodies may cause interference with the vaccine and the desired immune response to that vaccine." (Genovese *et al.*, page 5). Thus, Genovese *et al.* acknowledge exactly the technical problem that Applicants' methods are designed to overcome. Genovese *et al.*, however, adopt an approach to overcoming the problem that is different than Applicants' approach. Rather than attempting to influence the adaptive immune response, as Applicants do, Genovese *et al.* attempt to influence the non-specific innate immune response to compensate for the lack of effective adaptive immune response. Genovese *et al.* thus prime aspects of the non-specific innate immune response by administering immune lymphokines (ILKs). ILKs have no effect on the adaptive immune response that is the subject of Applicants' claims.

The Office Action directs Applicants' attention to page 3 of Genovese *et al.*, where it is asserted that "day old turkey poults were challenged with SE (Salmonella enteriditis). Vaccination with a foreign antigen will necessarily produce an innate as well as adaptive immune response." (Office Action, page 3). The Office Action further states, "[A] vaccination has already been given to the poults in the time required by the claimed invention. Thus, although Genovese et al. stimulate the immune systems of the bird post-vaccination, the vaccinations have none-the-less been administered[.]" This is incorrect.

First, Genovese *et al.* never vaccinate the poults. The SE challenge is not a vaccination; it is a challenge. Genovese *et al.* perform the challenge thirty minutes after priming the innate immune response in order to assess the extent to which the primed innate immune response protects the poults from challenge with the pathogen because Genovese *et al.* acknowledge that an adaptive immune response at this time is ineffective to protect the poult from infection.

Second, as is discussed in greater detail below, exposure to a foreign antigen does not "necessarily produce an innate as well as adaptive immune response" in embryos or newly hatched chicks. Exposure to a foreign antigen can result instead in immunological tolerance–the absence of an immune response.

For at least these reasons, Applicants respectfully submit that any rejection that depends, even only in part, on Genovese *et al.* fails to establish a *prima facie* of obviousness. Therefore, Applicants respectfully request that the rejection of claims 34, 37, 39-43, 67-69, 83-86, 89, 91-95, and 97-102 under 35 U.S.C. §103(a) as being unpatentable over Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766) in view of Genovese *et al.* (1998) in light of Sharma *et al.* (U.S. Patent No. 4,458,630) and the rejection of claims 34-44, 67-69, 71-82, and 84-102 under 35 U.S.C. §103(a) as being unpatentable over Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766) and further in view of Evans *et al.* (U.S. Patent No. 6,500,438) in view of Genovese *et al.* (1998) in light of Sharma *et al.* (U.S. Patent No. 4,458,630) be reconsidered and withdrawn.

Claims 34, 37, 39-43, 67-69, 83-86, 91-95, and 97-102 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Emery *et al.* (U.S. Patent No. 5,538,733) in view of Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766). Applicants respectfully traverse.

Claims 34-44, 67-69, 71-82, and 84-102 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766) in view of Emery *et al.* (U.S. Patent No. 5,538,733) and further in view of Evans *et al.* (U.S. Patent No. 6,500,438). Applicants respectfully traverse.

Claims 34, 69, and 84 are independent. Each of the remaining claims depends, directly or indirectly, from one of the independent claims and, therefore, includes all of the features recited in the independent claim and any intervening claim from which it depends. Thus, remarks that refer to one or more of the independent claims apply equally to any claim that depends from an identified independent claim.

Applicants respectfully submit that neither the combination of the '733 patent, the '479 patent, and the '766 patent nor the combination of the '479 patent, '766 patent, '733 patent, and '438 patent provide one skilled in the art with a reasonable expectation of success performing the methods recited in claims 34, 69, and 84.

The Office Action asserts that the '733 patent discusses the problem of vaccinating young animals because of the influence of maternal antibodies and proposes a solution involving administering vaccines present in a sustained and/or delayed delivery vehicle to young poultry at 1-90 days of age. (Office Action, page 13). The Office Action further asserts that the '733 patent teaches that the immunized poultry possess maternal antibody to the antigen and that SRPs are suitable immunogens for implantation. (Office Action, page 14). The Office Action acknowledges that the '733 patent fails to teach that the implant can be administered *in ovo*, the specific injection times recited in claims 39-42 and 44, the administration of a second dose of immunogen, or the inclusion of porins in the vaccine. (Office Action, page 15).

The Office Action further asserts that the '479 patent teaches a method of immunizing poultry using SRPs, and further teaches that SRPs can be administered using sustained release and, separately, that the SRPs can be delivered *in ovo*. (Office Action, page 16). Also, the '766 patent (Phelps *et al.*) is asserted to teach methods for introducing material into poultry eggs. (Office Action, page 17).

The Office Action asserts it would have been obvious to one skilled in the art to modify the method of sustained delivery of SRPs taught in the '733 patent by administering the SRPs *in ovo* according to the '479 patent. (Office Action, page 18). Applicants respectfully disagree.

Applicants disagree that one skilled in the art would have had a reasonable expectation that implanting a device for sustained release of a selected immunogen *in ovo* would successfully induce an effective adaptive immune response against the selected immunogen, as asserted in the Office Action. For the purposes of the current discussion, Applicants position need not rely on the technical feasibility of *in ovo* delivery methods as taught in the '766 patent, but rather rests on the knowledge of one skilled in the art that immunological tolerance can be induced by vaccinating animals with immature immune systems, particularly embryos. Nevertheless, Applicants reserve the right to argue that the teaching of Phelps *et al.* fails to permit one skilled in the art to successfully deliver a device for sustained release of a selected immunogen *in ovo*.

Immune tolerance to a foreign antigen can occur when a subject is exposed to a foreign antigen under conditions that elicit specific unresponsiveness to the foreign antigen rather than an adaptive humoral immune response to the antigen. In other words, under some circumstances, exposure to a foreign antigen does not necessarily result in the challenged subject mounting an adaptive immune response, but instead results in the subject's immune system perceiving the foreign antigen as "self" and establishing antigen-specific immune non-response.

Many of the conditions under which immune tolerance may be induced are present in the circumstance of *in ovo* vaccination as recited in Applicants' claims. (See, *Microbiology*, fourth edition, Davis *et al.* eds., 1990, J.B. Lippincott Co., Philadelphia, Pennsylvania, pp. 381-382.).

For example, SRPs–the immunogens specifically identified in the Office Action–are monomeric antigens, not aggregated; sustained release implants are not equivalent to injection into tissue, but are more similar to intravenous administration; and *in ovo* administration necessarily results in vaccination of the embryo rather than adult. So, one skilled in the art would recognize that vaccinating embryos or newly-hatched chicks using sustained release implants harbor the risk of inducing immune tolerance to the immunogen in the vaccine rather than raising adaptive immunity against the immunogen. Many of these conditions are present whether the sustained release implant is administered at one day of age (as in the '733 patent) or *in ovo*, as in the present claims.

The difference between vaccinating an egg by sustained release of a selected immunogen from a biocompatible implant at one day of age versus injecting the biocompatible implant *in ovo*–and a compelling reason why one skilled in the art would not extend the teaching of the '733 patent to *in ovo* delivery–is that the risk of inducing immune tolerance to the immunogen is greater when the biocompatible implant containing the immunogen is delivered *in ovo* compared to delivering the biocompatible implant after hatch. One reason for the increased risk of inducing immune tolerance when the biocompatible implant is injected *in ovo* is the different amounts of–and the corresponding effects of the different amounts of–circulating maternal antibody in the embryo versus in the newly-hatched chick.

Each of claims 34, 69, and 84 recites that the egg (or eggs) into which the biocompatible implant is injected comprises maternal antibody to the selected immunogen. Prior to hatch, some of the maternal antibody circulates in the embryo but most remains sequestered in the yolk. At hatching, however, the yolk is fully absorbed and the maternal antibody from the yolk is fully absorbed into the circulation of the chick. Thus, the chick–but not the embryo–has the full passive immunization benefit of the maternal antibodies. Consequently, the circulating maternal antibody environment is very different in the embryo than in the newly-hatched chick and this difference influences the risk of inducing immune tolerance.

As just explained, a day-old chick possesses the full amount of maternal antibody in circulation, including any maternal antibody specific to the selected immunogen. When a day-old chick is vaccinated with a biocompatible implant providing sustained release of the selected immunogen, maternal antibodies against the selected immunogen, if present, clear the immunogen from the chick's circulation without involving the chick's immature immune system, thereby reducing the risk of inducing immune tolerance to the selected immunogen. In contrast, when a biocompatible implant is provided *in ovo*, the level of maternal antibody absorbed by the chick–e.g., at day 20 of incubation as described in Example 4–is incomplete and, as a consequence, the embryo is at risk for developing immune tolerance to the immunogen in the biocompatible implant rather than adaptive immunity against the immunogen.

At least two factors put the embryo at greater risk for inducing immune tolerance to the selected immunogen than a newly-hatched chick receiving the very same sustained release implant. First, the embryo's immune system is less mature and, therefore, is less capable of raising an adaptive response to a foreign antigen and is more susceptible to inducing immune tolerance to the foreign antigen. Second, the immune system of an embryo is less protected from the foreign antigen by maternal antibodies, if present at all, than the immune system of a day old chick. Each factor, alone, is sufficient to render the effect of administering the sustained release implant to an embryo unpredictable. Taken together, however, one skilled in the art could not have predicted that vaccinating eggs using the recited implant would provide effective vaccination rather than inducing immune tolerance.

Thus, prior to Applicants' disclosure, it was unpredictable whether injecting a biocompatible implant containing a selected immunogen into an egg that possesses maternal antibody against the selected immunogen could induce an adaptive immune response against a selected immunogen or, alternatively, whether doing so would induce immune tolerance to the selected immunogen.

Because the combination of the '733 patent and the '479 patent fail to provide one skilled in the art with a reasonable expectation that injecting a biocompatible implant containing a

selected immunogen *in ovo* would induce an effective adaptive immune response rather than inducing immune tolerance, the combination does not help establish a *prima facie* case of obviousness against claims 34, 69, and 84. Nothing in Phelps *et al.* or Evans *et al.* cures this deficiency in the combined teachings of the '733 patent and the '479 patent.

As explained by Dr. Emery during the interview, the methods recited in claims 34, 69, and 84 permit those in the poultry industry to vaccinate a generation of eggs, at one time, and ensure that the resulting chicks can raise an adaptive immune response against a selected immunogen at the time–which can vary from egg to egg in a single generation from a single hen–when maternal antibody to the selected immunogen wanes. In the absence of such sustained release vaccination methods, those in the industry must otherwise vaccinated each chick every day over a multi-week period to ensure protection for the entire new generation because the waning of maternal antibody, if ever present at all, can vary from chick to chick.

Applicants respectfully submit that claims 34, 37, 39-43, 67-69, 83-86, 89, 91-95, and 97-102 are patentable under 35 U.S.C. §103(a) over Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766) in view of Genovese *et al.* (1998) in light of Sharma *et al.* (U.S. Patent No. 4,458,630); claims 34-44, 67-69, 71-82, and 84-102 are patentable under 35 U.S.C. §103(a) over Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766) and further in view of Evans *et al.* (U.S. Patent No. 6,500,438) in view of Genovese *et al.* (1998) in light of Sharma *et al.* (U.S. Patent No. 4,458,630); claims 34, 37, 39-43, 67-69, 83-86, 91-95, and 97-102 are patentable under 35 U.S.C. §103(a) over Emery *et al.* (U.S. Patent No. 5,538,733) in view of Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766); and claims 34-44, 67-69, 71-82, and 84-102 are patentable under 35 U.S.C. §103(a) over Emery *et al.* (U.S. Patent No. 5,830,479) in view of Phelps *et al.* (U.S. Patent No. 5,339,766) in view of Emery *et al.* (U.S. Patent No. 5,538,733) and further in view of Evans *et al.* (U.S. Patent No. 6,500,438). Accordingly, Applicants respectfully request that each of the pending rejections be reconsidered and withdrawn.

Summary

Applicants respectfully submit that the pending claims 34-44, 67-69, 71-82, and 84-102 are in condition for allowance and notification to that effect is respectfully requested. The Examiner is invited to contact Applicants' Representatives at the telephone number listed below if it is believed that prosecution of this application may be assisted thereby.

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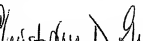
By: 
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Microbiology

Fourth Edition

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largely as soluble Ab–Ag complexes, which are taken up and degraded by macrophages. Free Ab appears at the end of the immune elimination stage.

Extensively phagocytized particulate Ags, such as bacteria and red cells, do not diffuse into extravascular spaces and hence do not exhibit the initial equilibration phase of rapid decrease in serum concentration after intravenous injection. Trace antigenic fragments can persist in lymphoid tissues long after the Ag is no longer detectable in blood.

Antigenic Competition. The response to an Ag may be diminished if an unrelated Ag is injected at the same time or shortly before. For instance, rabbits injected with a foreign serum (say from horse) produce Abs to serum globulin but not to serum albumin, although serum albumin alone elicits antialbumin Abs. Similarly, poly-L-alanyl-protein probably elicits Abs to the L-polypeptide, but on coimmunization with poly-D-alanyl-protein, only the latter elicits Abs to its polypeptide. This **intermolecular antigenic competition** is important in practical immunization programs (see Vaccination against Microbial Antigens, below), which often involve giving several different Ags in one vaccine. Adjusting the amounts of the several components ("balancing") can overcome the competitive effect.

Different determinants on the same molecule can also compete **(intramolecular competition):** thus a protein with both D- and L-polyalanyl peptides substituted on the same molecule evokes synthesis only of Abs to the D-polyalanyl groups. Presumably, the available cell surface receptors for the two determinants differ in affinity, and one determinant becomes dominant because the corresponding cells bind the limited supply of Ag. The mechanism for intermolecular competition is obscure.

INDUCED TOLERANCE TO FOREIGN ANTIGENS

This chapter noted earlier (see Tolerance of Self-Ags) that many foreign Ags can be administered under conditions that elicit specific unresponsiveness to that Ag, rather than Ab and T-cell responses to it. These conditions are described below.

Dose and Form of the Antigen. Every Ag has an optimal immunogenic dose range. Much larger amounts elicit tolerance ("high-zone" tolerance). With some Ags, lower amounts (repeatedly injected) can also cause tolerance ("low-zone" tolerance).

Tolerance was first induced experimentally with pneumococcal capsular polysaccharide, a T-independent Ag. Mice injected with 0.01 μg–1.0 μg of a pneumococcal polysaccharide, say of type 2, become resistant to infection with type 2 pneumococci and produce Abs to the polysaccharide, but if given 1000 μg of the same substance, they fail to become resistant or to form detectable Abs. The unresponsiveness is specific: although no response to type 2 can be elicited, the mice react normally to immunogenic doses of other Ags, including other pneumococcal polysaccharides.

Another factor with many Ags is their **physical state:** generally, aggregation enhances an Ag's immunogenicity, whereas monomeric Ags tend to be tolerogenic. **It is thus difficult or impossible to establish tolerance to many particulate Ags (viruses, bacteria, etc.),** which are usually highly immunogenic.

Route of administration is another determinant. Soluble Ags tend to be immunogenic when injected into tissues but to be tolerogenic when given intravenously.

Newborn versus Adult. In the fetus, and also shortly after birth in species where the newborn is relatively immature, tolerance is more easily established than in the adult. One reason seems to be that immature B cells are more readily made unresponsive than mature B cells because of **modulation** (see Clonal Deletion, below). As described later (Chapter 20), the effect of immaturity was first recognized by studies of foreign tissue grafts.

B Cells versus T Cells. Tolerance to **T-independent Ags** derives from unresponsiveness of the corresponding B cells, and T cells are not involved; thus T-deficient (e.g., athymic) mice are as easily made tolerant as normal mice by excessive doses of these Ags.

As noted previously (earlier, this chapter and also Chapter 15), in individuals who are naturally tolerant of a self-Ag, the cognate T cells are probably absent, whereas the corresponding B cells persist in an unresponsive state. However, when tolerance to a foreign Ag is induced experimentally, both T and B cells become unresponsive, but **T cells are made tolerant much more readily than B cells.** The difference is evident when the prospective donors of T and B cells are treated with various doses of Ag at various times before the cells are separately transferred to irradiated adoptive hosts (Fig. 16–17): compared with B cells, unresponsiveness of T cells is established sooner, lasts longer, and can be initiated by much lower levels of Ag.

How foreign Ags administered under tolerizing conditions cause inactivation (or elimination) of T cells is obscure. For B cells, the loss of cell-surface IgM may be a critical event (see IgM Modulation, above).

As noted earlier (Modulation of B-Cell Surface Ig, this chapter), the binding of a multivalent Ag (or of Ab–Ag complexes) by a B cell causes the cell's surface Ig to aggregate in the plane of the membrane. The resulting internalization (or shedding) of the aggregates leaves the cell denuded of its surface Ig and thus unresponsive to Ag until the Ig is regenerated.

In most B cells of mature mice, the modulation of surface Ig is slow (requiring about 1 h) and is seldom complete, and regeneration occurs in a few days. However, in immature B cells (from fetal or newborn mouse liver or spleen), the disappearance is more rapid and is

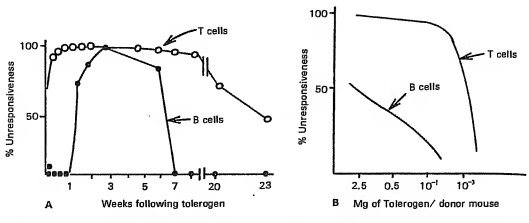

Figure 16–17. Induction of tolerance to a foreign Ag (bovine γ-globin, BγG). Thymus (T) and bone marrow (B) cells were removed at various times from mice rendered tolerant with various amounts of BγG and were tested, with complementary cells from normal donors, for the ability to cooperate in Ab formation when transferred to irradiated syngeneic mice. Results are given as percent of values in controls (untreated donors). In *A*, tolerance was induced with 2.5 mg of BγG. In *B*, the cells were removed 15–20 days after the doses of Ag shown (abscissa). Tolerance appeared sooner, lasted longer, and was established with lower Ag doses, in T than in B cells. (Chiller JM et al: Science 171:813, 1971)

complete, and regeneration may not occur at all. The difference suggests why experimental tolerance to foreign Ag is much more easily established in the fetus and newborn than in the adult. (The effect of immaturity on establishment of tolerance is discussed further in connection with Allograft Rejection, Chapter 20).

Persistence of Ag is necessary for the maintenance of tolerance. New B and T cells continue to be generated from stem cells, and once a tolerance-eliciting Ag disappears, responsiveness to it returns. Hence, the duration of tolerance depends on the amount of Ag injected, its rate of breakdown in tissues, and the rate at which new B and T cells of the appropriate specificity arise.

The duration of experimentally induced tolerance varies widely. Recovery is retarded by thymectomy (in the case of T-dependent Ags), and it is accelerated by the injection of lymphocytes from normally responsive syngeneic donors. Polysaccharides generally are not broken down in mammalian tissues (which lack the appropriate enzymes), and a single high dose of these Ags can establish life-long tolerance. Proteins, in contrast, are broken down readily; thus, a few weeks after tolerance to bovine serum albumin is established in mice, the declining level of Ag reaches an immunogenic range, causing a short burst of Ab (anti-BSA) synthesis.

VACCINATION AGAINST MICROBIAL ANTIGENS

Conventional vaccines are usually prepared from killed or attenuated microbes or from inactivated toxins (diphtheria and tetanus toxoids, Table 16–4): infectivity or toxicity is lost, while the epitopes that elicit protective Abs and/or T cells remain immunogenic. Given the effectiveness of these vaccines, the view that "Never in the history of human progress has a better or cheaper method of preventing illness been developed than immunization at its best" is probably justified. Nevertheless, serious side effects can be encountered, and although rare, they can be tragic (*e.g.*, about 1 in 300,000 children immunized with *B. pertussis* suffers serious brain damage). To reduce these hazards and to increase immunogenicity, various strategies are used to produce new vaccines.

1. Subunit Vaccines. Some of these consist of individual viral proteins, or segments of a protein, produced by recombinant DNA technology; others consist of synthetic short peptides that encompass the epitopes that elicit protective Abs. Thus, an effective vaccine against hepatitis B virus consists of just the virus's envelope glycoprotein. In another one, a small synthetic peptide (12 amino acid residues), encompassing the key epitope of the infectious (sporozoite) form of malaria, is linked covalently to tetanus toxoid as carrier protein; Abs elicited by the conjugate protect mice against infection with malaria organisms.

2. Polysaccharide–Protein Conjugates. Vaccines consisting of purified polysaccharides from pneumococci and *Haemophilus influenzae* type b (Hib) elicit Abs that protect against infection by the corresponding bacteria. In an improved vaccine, Hib polysaccharide is linked covalently to a protein (tetanus toxoid), converting the T-independent Ag (polysaccharide alone) into a T-dependent one. The benefits are evident in young chil-

RELATED PROCEEDINGS APPENDIX

Serial No. 10/749,602

Docket No. 293.00010102

There are no Appeals or Interferences known to Appellants' Representatives which would directly affect, be directly affected by, or have a bearing on the Board's decision in the pending Appeal.

APPENDIX OF CITED CASE LAW

Serial No. 10/749,602

Docket No. 293.00010102

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1. KSR International Co. v. Teleflex Inc., 127 SCt 1727, 167 LEd2d 705, 550 US 398, 82 USPQ2d 1385 (U.S. 2007).

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KSR International Co. v. Teleflex Inc., 82 USPQ2d 1385 (U.S. 2007)

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KSR International Co. v. Teleflex Inc.
U.S. Supreme Court
No. 04-1350
Decided April 30, 2007
127 SCt 1727
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550 US 398

Headnotes

PATENTS

[1] Patentability/Validity — Obviousness — Combining references (▶115.0905)

Rigid application of "teaching, suggestion, or motivation" test, under which patent claim is proved obvious only if prior art, nature of problem addressed by inventor, or knowledge of person having ordinary skill in art reveals some motivation or suggestion to combine prior art teachings, is inconsistent with expansive and flexible "functional approach" to resolution of obviousness issue, under which scope and content of prior art are determined, differences between prior art and claims at issue are ascertained, level of ordinary skill in pertinent art is resolved, and secondary considerations such as commercial success, long felt but unsolved needs, and failure of others may be considered if doing so would prove instructive; rigid TSM approach is therefore rejected.

[2] Patentability/Validity — Obviousness — Combining references (▶115.0905)

Patentability/Validity — Obviousness — Evidence of (▶115.0906)

Variations of particular work available in one field of endeavor may be prompted by design incentives and other market forces, either in same field or different one, and if person of ordinary skill in art can implement predictable variation, 35 U.S.C. § 103 likely bars its patentability; similarly, if particular technique has been used to improve one device, and person of ordinary skill would recognize that it would improve similar devices in same way, then using that technique is obvious unless its actual application is beyond person's skill, and court resolving obviousness issue therefore must ask whether improvement is more than predictable use of prior art elements according to their established functions.

[3] Patentability/Validity — Obviousness — Combining references (▶115.0905)

Patentability/Validity — Obviousness — Evidence of (▶115.0906)

Court determining whether claimed combination of elements known in prior art would have been obvious will often be required to look to interrelated teachings of multiple patents, effects of demands known to design community or present in marketplace, and background knowledge of person of ordinary skill in art in order to determine whether there was apparent reason to combine known elements in manner claimed in patent in suit, and in order to facilitate review, this analysis should be made explicit; however, such analysis need not seek out precise teachings directed to specific subject matter of challenged claim, since court can take account of inferences and creative steps that person of ordinary skill in art would employ.

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[4] Patentability/Validity — Obviousness — Combining references (▶115.0905)

Idea underlying "teaching, suggestion, or motivation" test, under which patent claim is proved obvious only if prior art, nature of problem addressed by inventor, or knowledge of person having ordinary skill in art reveals some motivation or suggestion to combine prior art teachings, is not necessarily inconsistent with expansive and flexible "functional approach" to resolution of obviousness issue, since TSM test is based on helpful insights, namely, that patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in prior art, and that it can be important to identify reason that would have prompted person of ordinary skill in art to combine elements in manner claimed by new invention; however, it is error to apply TSM test as rigid and mandatory formula that limits obviousness analysis through formalistic conception of words "teaching," "suggestion,"and "motivation," or by overemphasis on importance of published articles and explicit content of issued patents, since market demand, rather than scientific literature, often drives design trends, and granting patent protection to advances that would occur "in the ordinary course" without real innovation retards progress and may, in case of patents

combining previously known elements, deprive prior inventions of their value or utility.

[5] Patentability/Validity — Obviousness — Combining references (▶115.0905)

Narrow conception of obviousness inquiry, reflected in appellate court's application of "teaching, suggestion, or motivation" test, resulted in erroneous conclusion that summary judgment of obviousness should be vacated, since decision was based on erroneous holding that courts and patent examiners should look only to problem that patentee was trying to solve, and on erroneous assumption that person of ordinary skill in art attempting to solve problem will be led only to those elements of prior art designed to solve same problem, since court erroneously concluded that patent claim cannot be proved obvious merely by showing that combination of elements was "obvious to try," and since appellate court drew wrong conclusion from risk of courts and patent examiners falling prey to "hindsight" bias, in that rigid application of preventative rules that deny fact finders recourse to common sense are neither necessary nor consistent with precedent.

[6] Patentability/Validity — Obviousness — Combining references (▶115.0905)

Patentability/Validity — Obviousness — Evidence of (▶115.0906)

Fact that claimed combination of elements was "obvious to try" might show that such combination was obvious under 35 U.S.C. § 103, since, if there is design need or market pressure to solve problem, and there are finite number of identified, predictable solutions, person of ordinary skill in art has good reason to pursue known options within his or her technical grasp, and if this leads to anticipated success, it is likely product of ordinary skill and common sense, not innovation.

[7] Patentability/Validity — Obviousness — Relevant prior art — Particular inventions (▶115.0903.03)

Patentability/Validity — Obviousness — Combining references (▶115.0905)

Asserted claim of patent for position-adjustable vehicle pedal assembly having electronic pedal-position sensor attached to fixed pivot point is invalid as obvious over combination of prior art references, since prior art patent discloses support structure for adjustable pedal assembly in which one pivot point stays fixed, since, at relevant time, marketplace had created strong incentive to convert mechanical pedals to those employing electronic sensors, and pedal designer of ordinary skill would have seen benefit in upgrading device of prior patent with sensor required by new engines using computer-controlled throttles, since other prior art references taught utility of placing sensor on pedal's support structure rather than on footpad, and on nonmoving part of pedal structure, since most obvious nonmoving point on structure from

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which sensor can easily detect pedal position is fixed pivot point, and since designer seeking to avoid wire-chafing problems with electronic adjustable pedals would have known to employ adjustable pedal with fixed pivot disclosed by prior art patent; declaration submitted by patentees does not indicate that device of prior patent was so flawed that there was no reason to upgrade it to be compatible with modern engines, and patentees have shown no secondary considerations to dislodge obviousness determination.

[8] Patentability/Validity — Obviousness — Evidence of (▶115.0906)

JUDICIAL PRACTICE AND PROCEDURE

Procedure — Summary judgment — Patents (▶410.3303)

Procedure — Evidence — Expert testimony (▶410.3703)

Party's submission of conclusory expert affidavit addressing issue of obviousness in patent action does not preclude summary judgment, even though federal district court can and should take into account expert testimony, which may resolve or keep open certain questions of fact, since ultimate judgment of obviousness is legal determination; in present case, in which content of prior art, scope of asserted claim, and level of ordinary skill in art were not in material dispute, and obviousness of claim was apparent from these factors, summary judgment was appropriate, and nothing in declarations proffered by patentees prevented district court from reaching conclusions underlying its order for summary judgment of obviousness.

Particular Patents

Particular patents — General and mechanical —Vehicle control pedal assembly

6,237,565, Engelgau, adjustable pedal assembly with electronic throttle control, invalid for obviousness.

Case History and Disposition

On writ of certiorari to the U.S. Court of Appeals for the Federal Circuit, Schall, J.

Action by Teleflex Inc. and Technology Holding Co. against KSR International Co. for patent infringement. The U.S. District Court for the Eastern District of Michigan granted summary judgment in favor of defendant on ground that patent in suit was invalid for obviousness, and plaintiffs appealed. Grant of summary judgment was vacated and remanded, and defendant-appellee filed petition for writ of certiorari. Reversed and remanded.

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Syllabus

Syllabus by the Court.

To control a conventional automobile's speed, the driver depresses or releases the gas pedal, which interacts with the throttle via a cable or other mechanical link. Because the pedal's position in the footwell normally cannot be adjusted, a driver wishing to be closer or farther from it must either reposition himself in the seat or move the seat, both of which can be imperfect solutions for smaller drivers in cars with deep footwells. This prompted inventors to design and patent pedals that could be adjusted to change their locations. The Asano patent reveals a support structure whereby, when the pedal location is adjusted, one of the pedal's pivot points stays fixed. Asano is also designed so that the force necessary to depress the pedal is the same regardless of location adjustments. The Redding patent reveals a different, sliding mechanism where both the pedal and the pivot point are adjusted.

In newer cars, computer-controlled throttles do not operate through force transferred from the pedal by a mechanical link, but open and close valves in response to electronic signals. For the computer to know what is happening with the pedal, an electronic sensor must translate the mechanical operation into digital data. Inventors had obtained a number of patents for such sensors. The so-called '936 patent taught that it was preferable to detect the pedal's position in the pedal mechanism, not in the engine, so the patent disclosed a pedal with an electronic sensor on a pivot point in the pedal assembly. The Smith patent taught that to prevent the wires connecting the sensor to the computer from chafing and wearing out, the sensor should be put on a fixed part of the pedal assembly rather than in or on the pedal's footpad. Inventors had also patented self-contained modular sensors, which can be taken off the shelf and attached to any mechanical pedal to allow it to function with a computer-controlled throttle. The '068 patent disclosed one such sensor. Chevrolet also manufactured trucks using modular sensors attached to the pedal support bracket, adjacent to the pedal and engaged with the pivot shaft about which the pedal rotates. Other patents disclose electronic sensors attached to adjustable pedal assemblies. For example, the Rixon patent locates the sensor in the pedal footpad, but is known for wire chafing.

After petitioner KSR developed an adjustable pedal system for cars with cable-actuated throttles and obtained its '976 patent for the design, General Motors Corporation (GMC) chose KSR to supply adjustable pedal systems for trucks using computer-controlled throttles. To make the '976 pedal compatible with the trucks, KSR added a modular sensor to its design. Respondents (Teleflex) hold the exclusive license for the Engelgau patent, claim 4 of which discloses a position-adjustable pedal assembly with an electronic pedal position sensor attached at a fixed pivot point. Despite having denied a similar, broader claim, the

U.S. Patent and Trademark Office (PTO) had allowed claim 4 because it included the limitation of a fixed pivot position, which distinguished the design from Redding's. Asano was neither included among the Engelgau patent's prior art references nor mentioned in the patent's prosecution, and the PTO did not have before it an adjustable pedal with a fixed pivot point. After learning of KSR's design for GMC, Teleflex sued for infringement, asserting that KSR's pedal system infringed the Engelgau patent's claim 4. KSR countered that claim 4 was invalid under § 103 of the Patent Act, which forbids issuance of a patent when "the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art."

Graham v. *John Deere Co. of Kansas City*, 383 U.S. 1, 17–18 [148 USPQ 459], set out an objective analysis for applying § 103: "[T]he scope and content of the prior art are ... determined; differences between the prior art and the claims at issue are ... ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs,

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failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." While the sequence of these questions might be reordered in any particular case, the factors define the controlling inquiry. However, seeking to resolve the obviousness question with more uniformity and consistency, the Federal Circuit has employed a "teaching, suggestion, or motivation" (TSM) test, under which a patent claim is only proved obvious if the prior art, the problem's nature, or the knowledge of a person having ordinary skill in the art reveals some motivation or suggestion to combine the prior art teachings.

The District Court granted KSR summary judgment. After reviewing pedal design history, the Engelgau patent's scope, and the relevant prior art, the court considered claim 4's validity, applying *Graham*'s framework to determine whether under summary-judgment standards KSR had demonstrated that claim 4 was obvious. The court found "little difference" between the prior art's teachings and claim 4: Asano taught everything contained in the claim except using a sensor to detect the pedal's position and transmit it to a computer controlling the throttle. That additional aspect was revealed in, e.g., the '068 patent and Chevrolet's sensors. The court then held that KSR satisfied the TSM test, reasoning (1) the state of the industry would lead inevitably to combinations of electronic sensors and adjustable pedals, (2) Rixon provided the basis for these developments, and (3)Smith taught a solution to Rixon's chafing problems by positioning the sensor on the pedal's fixed structure, which could lead to the combination of a pedal like Asano with a pedal position sensor.

Reversing, the Federal Circuit ruled the District Court had not applied the TSM test strictly enough, having failed to make findings as to the specific understanding or principle within a skilled artisan's knowledge that would have motivated one with no knowledge of the invention to attach an electronic control to the Asano assembly's support bracket. The Court of Appeals held that the District Court's recourse to the nature of the problem to be solved was insufficient because, unless the prior art references addressed the precise problem that the patentee was trying to solve, the problem would not motivate an inventor to look at those references. The appeals court found that the Asano pedal was designed to ensure that the force required to depress the pedal is the same no matter how the pedal is adjusted, whereas Engelgau sought to provide a simpler, smaller, cheaper adjustable electronic pedal. The Rixon pedal, said the court, suffered from chafing but was not designed to solve that problem and taught nothing helpful to Engelgau's purpose. Smith, in turn, did not relate to adjustable pedals and did not necessarily go to the issue of motivation to attach the electronic control on the pedal assembly's support bracket. So interpreted, the court held, the patents would not have led a person of ordinary skill to put a sensor on an Asano-like pedal. That it might have been obvious to try that combination was likewise irrelevant. Finally, the court held that genuine issues of material fact precluded summary judgment.

Held: The Federal Circuit addressed the obviousness question in a narrow, rigid manner that is inconsistent with § 103 and this Court's precedents. KSR provided convincing evidence that mounting an available sensor on a

fixed pivot point of the Asano pedal was a design step well within the grasp of a person of ordinary skill in the relevant art and that the benefit of doing so would be obvious. Its arguments, and the record, demonstrate that the Engelgau patent's claim 4 is obvious. Pp. 11–24.

1. *Graham* provided an expansive and flexible approach to the obviousness question that is inconsistent with the way the Federal Circuit applied its TSM test here. Neither § 103's enactment nor *Graham*'s analysis disturbed the Court's earlier instructions concerning the need for caution in granting a patent based on the combination of elements found in the prior art. See *Great Atlantic & Pacific Tea Co.* v. *Supermarket Equipment Corp.*, 340 U.S. 147, 152 [87 USPQ 303]. Such a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results. See, e.g., *United States* v. *Adams*, 383 U.S. 39, 50–52 [148 USPQ 479]. When a work is available in one field, design incentives and other market forces can prompt variations of it, either in the same field or in another. If a person of ordinary skill in the art can implement a predictable variation, and would see the benefit of doing so, § 103 likely bars its patentability. Moreover, if a technique has been

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used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond that person's skill. A court must ask whether the improvement is more than the predictable use of prior-art elements according to their established functions. Following these principles may be difficult if the claimed subject matter involves more than the simple substitution of one known element for another or the mere application of a known technique to a piece of prior art ready for the improvement. To determine whether there was an apparent reason to combine the known elements in the way a patent claims, it will often be necessary to look to interrelated teachings of multiple patents; to the effects of demands known to the design community or present in the marketplace; and to the background knowledge possessed by a person having ordinary skill in the art. To facilitate review, this analysis should be made explicit. But it need not seek out precise teachings directed to the challenged claim's specific subject matter, for a court can consider the inferences and creative steps a person of ordinary skill in the art would employ. Pp. 11–14.

(b)The TSM test captures a helpful insight: A patent composed of several elements is not proved obvious merely by demonstrating that each element was, independently, known in the prior art. Although common sense directs caution as to a patent application claiming as innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the art to combine the elements as the new invention does. Inventions usually rely upon building blocks long since uncovered, and claimed discoveries almost necessarily will be combinations of what, in some sense, is already known. Helpful insights, however, need not become rigid and mandatory formulas. If it is so applied, the TSM test is incompatible with this Court's precedents. The diversity of inventive pursuits and of modern technology counsels against confining the obviousness analysis by a formalistic conception of the words teaching, suggestion, and motivation, or by overemphasizing the importance of published articles and the explicit content of issued patents. In many fields there may be little discussion of obvious techniques or combinations, and market demand, rather than scientific literature, may often drive design trends. Granting patent protection to advances that would occur in the ordinary course without real innovation retards progress and may, for patents combining previously known elements, deprive prior inventions of their value or utility. Since the TSM test was devised, the Federal Circuit doubtless has applied it in accord with these principles in many cases. There is no necessary inconsistency between the test and the *Graham* analysis. But a court errs where, as here, it transforms general principle into a rigid rule limiting the obviousness inquiry. Pp. 14–15.

(c)The flaws in the Federal Circuit's analysis relate mostly to its narrow conception of the obviousness inquiry consequent in its application of the TSM test. The Circuit first erred in holding that courts and patent examiners should look only to the problem the patentee was trying to solve. Under the correct analysis, any need or problem known in the field and addressed by the patent can provide a reason for combining the elements in the

manner claimed. Second, the appeals court erred in assuming that a person of ordinary skill in the art attempting to solve a problem will be led only to those prior art elements designed to solve the same problem. The court wrongly concluded that because Asano's primary purpose was solving the constant ratio problem, an inventor considering how to put a sensor on an adjustable pedal would have no reason to consider putting it on the Asano pedal. It is common sense that familiar items may have obvious uses beyond their primary purposes, and a person of ordinary skill often will be able to fit the teachings of multiple patents together like pieces of a puzzle. Regardless of Asano's primary purpose, it provided an obvious example of an adjustable pedal with a fixed pivot point, and the prior art was replete with patents indicating that such a point was an ideal mount for a sensor. Third, the court erred in concluding that a patent claim cannot be proved obvious merely by showing that the combination of elements was obvious to try. When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. Finally, the court drew the wrong conclusion from the risk of courts and patent examiners falling prey to hindsight bias. Rigid preventative

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rules that deny recourse to common sense are neither necessary under, nor consistent with, this Court's case law. Pp. 15–18.

2. Application of the foregoing standards demonstrates that claim 4 is obvious. Pp. 18–23.

(a)The Court rejects Teleflex's argument that the Asano pivot mechanism's design prevents its combination with a sensor in the manner claim 4 describes. This argument was not raised before the District Court, and it is unclear whether it was raised before the Federal Circuit. Given the significance of the District Court's finding that combining Asano with a pivot-mounted pedal position sensor fell within claim 4's scope, it is apparent that Teleflex would have made clearer challenges if it intended to preserve this claim. Its failure to clearly raise the argument, and the appeals court's silence on the issue, lead this Court to accept the District Court's conclusion. Pp. 18–20.

(b)The District Court correctly concluded that when Engelgau designed the claim 4 subject matter, it was obvious to a person of ordinary skill in the art to combine Asano with a pivot-mounted pedal position sensor. There then was a marketplace creating a strong incentive to convert mechanical pedals to electronic pedals, and the prior art taught a number of methods for doing so. The Federal Circuit considered the issue too narrowly by, in effect, asking whether a pedal designer writing on a blank slate would have chosen both Asano and a modular sensor similar to the ones used in the Chevrolet trucks and disclosed in the '068 patent. The proper question was whether a pedal designer of ordinary skill in the art, facing the wide range of needs created by developments in the field, would have seen an obvious benefit to upgrading Asano with a sensor. For such a designer starting with Asano, the question was where to attach the sensor. The '936 patent taught the utility of putting the sensor on the pedal device. Smith, in turn, explained not to put the sensor on the pedal footpad, but instead on the structure. And from Rixon's known wire-chafing problems, and Smith's teaching that the pedal assemblies must not precipitate any motion in the connecting wires, the designer would know to place the sensor on a nonmoving part of the pedal structure. The most obvious such point is a pivot point. The designer, accordingly, would follow Smith in mounting the sensor there. Just as it was possible to begin with the objective to upgrade Asano to work with a computer-controlled throttle, so too was it possible to take an adjustable electronic pedal like Rixon and seek an improvement that would avoid the wire-chafing problem. Teleflex has not shown anything in the prior art that taught away from the use of Asano, nor any secondary factors to dislodge the determination that claim 4 is obvious. Pp. 20–23.

3. The Court disagrees with the Federal Circuit's holding that genuine issues of material fact precluded summary judgment. The ultimate judgment of obviousness is a legal determination. *Graham*, 383 U.S., at 17. Where, as here, the prior art's content, the patent claim's scope, and the level of ordinary skill in the art are not in material dispute and the claim's obviousness

is apparent, summary judgment is appropriate. P. 23.

119 Fed. Appx. 282, reversed and remanded.

Kennedy, J., delivered the opinion for a unanimous Court.

Opinion Text

Opinion By:

Kennedy, J.

Teleflex Incorporated and its subsidiary Technology Holding Company—both referred to here as Teleflex—sued KSR International Company for patent infringement. The patent at issue, United States Patent No. 6,237,565 B1, is entitled "Adjustable Pedal Assembly With Electronic Throttle Control."

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Supplemental App. 1. The patentee is Steven J. Engelgau, and the patent is referred to as "the Engelgau patent." Teleflex holds the exclusive license to the patent.

Claim 4 of the Engelgau patent describes a mechanism for combining an electronic sensor with an adjustable automobile pedal so the pedal's position can be transmitted to a computer that controls the throttle in the vehicle's engine. When Teleflex accused KSR of infringing the Engelgau patent by adding an electronic sensor to one of KSR's previously designed pedals, KSR countered that claim 4 was invalid under the Patent Act, 35 U.S.C. § 103, because its subject matter was obvious.

Section 103 forbids issuance of a patent when "the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains."

In *Graham* v. *John Deere Co. of Kansas City*, 383 U.S. 1 [148 USPQ 459] (1966), the Court set out a framework for applying the statutory language of § 103, language itself based on the logic of the earlier decision in *Hotchkiss* v. *Greenwood*, 11 How. 248 (1851), and its progeny. See 383 U.S., at 15–17. The analysis is objective:

> "Under § 103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained;and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." *Id.*, at 17–18.

While the sequence of these questions might be reordered in any particular case, the factors continue to define the inquiry that controls. If a court, or patent examiner, conducts this analysis and concludes the claimed subject matter was obvious, the claim is invalid under § 103.

Seeking to resolve the question of obviousness with more uniformity and consistency, the Court of Appeals for the Federal Circuit has employed an approach referred to by the parties as the "teaching, suggestion, or motivation" test (TSM test), under which a patent claim is only proved obvious if "some motivation or suggestion to combine the prior art teachings" can be found in the prior art, the nature of the problem, or the knowledge of a person having ordinary skill in the art. See, *e.g., Al-Site Corp.* v. *VSI Int'l, Inc.*, 174 F.3d 1308, 1323–1324 [50 USPQ2d 1161] (CA Fed. 1999). KSR challenges that test, or at least its application in this case. See 119 Fed. Appx. 282, 286–290 (CA Fed. 2005). Because the Court of Appeals addressed the question of obviousness in a manner contrary to § 103 and our precedents, we granted certiorari, 547 U.S. ___ (2006). We now reverse.

I

A

In car engines without computer-controlled throttles, the accelerator pedal interacts with the throttle via cable or other mechanical link. The pedal arm acts as a lever rotating around a pivot point. In a cable-actuated throttle control the rotation caused by pushing down the pedal pulls a cable, which in turn pulls open valves in the carburetor or fuel injection unit. The wider the valves open, the more fuel and air are released, causing combustion to increase and the car to accelerate. When the driver takes his foot off the pedal, the opposite occurs as the cable is released and the valves slide closed.

In the 1990's it became more common to install computers in cars to control engine operation. Computer-controlled throttles open and close valves in response to electronic signals, not through force transferred from the pedal by a mechanical link. Constant, delicate

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adjustments of air and fuel mixture are possible. The computer's rapid processing of factors beyond the pedal's position improves fuel efficiency and engine performance.

For a computer-controlled throttle to respond to a driver's operation of the car, the computer must know what is happening with the pedal. A cable or mechanical link does not suffice for this purpose; at some point, an electronic sensor is necessary to translate the mechanical operation into digital data the computer can understand.

Before discussing sensors further we turn to the mechanical design of the pedal itself. In the traditional design a pedal can be pushed down or released but cannot have its position in the footwell adjusted by sliding the pedal forward or back. As a result, a driver who wishes to be closer or farther from the pedal must either reposition himself in the driver's seat or move the seat in some way. In cars with deep footwells these are imperfect solutions for drivers of smaller stature. To solve the problem, inventors, beginning in the 1970's, designed pedals that could be adjusted to change their location in the footwell. Important for this case are two adjustable pedals disclosed in U.S. Patent Nos. 5,010,782 (filed July 28, 1989) (Asano) and 5,460,061(filed Sept. 17, 1993) (Redding). The Asano patent reveals a support structure that houses the pedal so that even when the pedal location is adjusted relative to the driver, one of the pedal's pivot points stays fixed. The pedal is also designed so that the force necessary to push the pedal down is the same regardless of adjustments to its location. The Redding patent reveals a different, sliding mechanism where both the pedal and the pivot point are adjusted.

We return to sensors. Well before Engelgau applied for his challenged patent, some inventors had obtained patents involving electronic pedal sensors for computer-controlled throttles. These inventions, such as the device disclosed in U.S. Patent No. 5,241,936 (filed Sept. 9, 1991) ('936), taught that it was preferable to detect the pedal's position in the pedal assembly, not in the engine. The '936 patent disclosed a pedal with an electronic sensor on a pivot point in the pedal assembly. U.S. Patent No. 5,063,811 (filed July 9, 1990)(Smith) taught that to prevent the wires connecting the sensor to the computer from chafing and wearing out, and to avoid grime and damage from the driver's foot, the sensor should be put on a fixed part of the pedal assembly rather than in or on the pedal's footpad.

In addition to patents for pedals with integrated sensors inventors obtained patents for self-contained modular sensors. A modular sensor is designed independently of a given pedal so that it can be taken off the shelf and attached to mechanical pedals of various sorts, enabling the pedals to be used in automobiles with computer-controlled throttles. One such sensor was disclosed in U.S. Patent No. 5,385,068 (filed Dec. 18, 1992) ('068). In 1994, Chevrolet manufactured a line of trucks using modular sensors "attached to the pedal support bracket, adjacent to the pedal and engaged with the pivot shaft about which the pedal rotates in operation." 298 F.Supp.2d 581, 589(ED Mich. 2003).

The prior art contained patents involving the placement of sensors on adjustable pedals as well. For example, U.S. Patent No. 5,819,593(filed Aug. 17, 1995) (Rixon) discloses an adjustable pedal assembly with an electronic sensor for detecting the pedal's position. In the Rixon pedal the sensor is located in the pedal footpad. The Rixon pedal was known to suffer from wire chafing when the pedal was depressed and released.

This short account of pedal and sensor technology leads to the instant case.

B

KSR, a Canadian company, manufactures and supplies auto parts, including pedal systems. Ford Motor Company hired KSR in 1998 to supply an adjustable pedal system for various lines of automobiles with cable-actuated throttle controls. KSR developed an adjustable mechanical pedal for Ford and obtained U.S. Patent No. 6,151,976 (filed July 16, 1999)('976) for the design. In 2000, KSR was chosen by General Motors Corporation (GMC or GM) to supply adjustable pedal systems for Chevrolet and GMC light trucks that used engines with computer-controlled throttles. To make the '976 pedal compatible with the trucks,

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KSR merely took that design and added a modular sensor.

Teleflex is a rival to KSR in the design and manufacture of adjustable pedals. As noted, it is the exclusive licensee of the Engelgau patent. Engelgau filed the patent application on August 22, 2000 as a continuation of a previous

application for U.S. Patent No. 6,109,241, which was filed on January 26, 1999. He has sworn he invented the patent's subject matter on February 14, 1998. The Engelgau patent discloses an adjustable electronic pedal described in the specification as a "simplified vehicle control pedal assembly that is less expensive, and which uses fewer parts and is easier to package within the vehicle." Engelgau, col. 2, lines 2–5, Supplemental App. 6. Claim 4 of the patent, at issue here, describes:

> "A vehicle control pedal apparatus comprising:
>
> a support adapted to be mounted to a vehicle structure;
>
> an adjustable pedal assembly having a pedal arm moveable in for[e] and aft directions with respect to said support;
>
> a pivot for pivotally supporting said adjustable pedal assembly with respect to said support and defining a pivot axis; and
>
> an electronic control attached to said support for controlling a vehicle system;
>
> said apparatus characterized by said electronic control being responsive to said pivot for providing a signal that corresponds to pedal arm position as said pedal arm pivots about said pivot axis between rest and applied positions wherein the position of said pivot remains constant while said pedal arm moves in fore and aft directions with respect to said pivot." *Id.*, col. 6, lines 17–36, Supplemental App. 8 (diagram numbers omitted).

We agree with the District Court that the claim discloses "a position-adjustable pedal assembly with an electronic pedal position sensor attached to the support member of the pedal assembly. Attaching the sensor to the support member allows the sensor to remain in a fixed position while the driver adjusts the pedal." 298 F.Supp.2d, at 586–587.

Before issuing the Engelgau patent the U.S. Patent and Trademark Office (PTO)rejected one of the patent claims that was similar to, but broader than, the present claim 4. The claim did not include the requirement that the sensor be placed on a fixed pivot point. The PTO concluded the claim was an obvious combination of the prior art disclosed in Redding and Smith, explaining:

> " 'Since the prior ar[t] references are from the field of endeavor, the purpose disclosed ... would have been recognized in the pertinent art of Redding. Therefore it would have been obvious ... to provide the device of Redding with the ... means attached to a support member as taught by Smith.' " *Id.*, at 595.

In other words Redding provided an example of an adjustable pedal and Smith explained how to mount a sensor on a pedal's support structure, and the rejected patent claim merely put these two teachings together.

Although the broader claim was rejected, claim 4 was later allowed because it included the limitation of a fixed pivot point, which distinguished the design from Redding's. *Ibid.* Engelgau had not included Asano among the prior art references, and Asano was not mentioned in the patent's prosecution. Thus, the PTO did not have before it an adjustable pedal with a fixed pivot point. The patent issued on May 29, 2001 and was assigned to Teleflex.

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Upon learning of KSR's design for GM, Teleflex sent a warning letter informing KSR that its proposal would violate the Engelgau patent. " 'Teleflex believes that any supplier of a product that combines an adjustable pedal with an electronic throttle control necessarily employs technology covered by one or more' " of Teleflex's patents. *Id.*, at 585. KSR refused to enter a royalty arrangement with Teleflex; so Teleflex sued for infringement, asserting KSR's pedal infringed the Engelgau patent and two other patents. *Ibid.* Teleflex later abandoned its claims regarding the other patents and dedicated the patents to the public. The remaining contention was that KSR's pedal system for GM infringed claim 4 of the Engelgau patent. Teleflex has not argued that the other three claims of the patent are infringed by KSR's pedal, nor has Teleflex argued that the mechanical adjustable pedal designed by KSR for Ford infringed any of its patents.

C

The District Court granted summary judgment in KSR's favor. After reviewing the pertinent history of pedal design, the scope of the Engelgau patent, and the relevant prior art, the court considered the validity of the contested claim. By direction of 35 U.S.C. § 282, an issued patent is presumed valid. The District Court applied *Graham*'s framework to determine

whether under summary-judgment standards KSR had overcome the presumption and demonstrated that claim 4 was obvious in light of the prior art in existence when the claimed subject matter was invented. See § 102(a).

The District Court determined, in light of the expert testimony and the parties' stipulations, that the level of ordinary skill in pedal design was " 'an undergraduate degree in mechanical engineering (or an equivalent amount of industry experience) [and]familiarity with pedal control systems for vehicles.' "298 F.Supp.2d, at 590. The court then set forth the relevant prior art, including the patents and pedal designs described above.

Following *Graham*'s direction, the court compared the teachings of the prior art to the claims of Engelgau. It found "little difference." 298 F.Supp.2d, at 590. Asano taught everything contained in claim 4 except the use of a sensor to detect the pedal's position and transmit it to the computer controlling the throttle. That additional aspect was revealed in sources such as the '068 patent and the sensors used by Chevrolet.

Under the controlling cases from the Court of Appeals for the Federal Circuit, however, the District Court was not permitted to stop there. The court was required also to apply the TSM test. The District Court held KSR had satisfied the test. It reasoned (1) the state of the industry would lead inevitably to combinations of electronic sensors and adjustable pedals, (2) Rixon provided the basis for these developments, and (3)Smith taught a solution to the wire chafing problems in Rixon, namely locating the sensor on the fixed structure of the pedal. This could lead to the combination of Asano, or a pedal like it, with a pedal position sensor.

The conclusion that the Engelgau design was obvious was supported, in the District Court's view, by the PTO's rejection of the broader version of claim 4. Had Engelgau included Asano in his patent application, it reasoned, the PTO would have found claim 4 to be an obvious combination of Asano and Smith, as it had found the broader version an obvious combination of Redding and Smith. As a final matter, the District Court held that the secondary factor of Teleflex's commercial success with pedals based on Engelgau's design did not alter its conclusion. The District Court granted summary judgment for KSR.

With principal reliance on the TSM test, the Court of Appeals reversed. It ruled the District Court had not been strict enough in applying the test, having failed to make " 'finding[s] as to the specific understanding or principle within the knowledge of a skilled artisan that would have motivated one with no knowledge of [the] invention' ... to attach an electronic control to the support bracket of the Asano assembly." 119 Fed. Appx., at 288 (brackets in original) (quoting *In re Kotzab*, 217 F.3d 1365, 1371 [55 USPQ2d 1313] (CA Fed. 2000)). The Court of Appeals held that the District Court was incorrect that the nature of the problem

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to be solved satisfied this requirement because unless the "prior art references address[ed]the precise problem that the patentee was trying to solve,"the problem would not motivate an inventor to look at those references. 119 Fed. Appx., at 288.

Here, the Court of Appeals found, the Asano pedal was designed to solve the " 'constant ratio problem' "—that is, to ensure that the force required to depress the pedal is the same no matter how the pedal is adjusted—whereas Engelgau sought to provide a simpler, smaller, cheaper adjustable electronic pedal. *Ibid.* As for Rixon, the court explained, that pedal suffered from the problem of wire chafing but was not designed to solve it. In the court's view Rixon did not teach anything helpful to Engelgau's purpose. Smith, in turn, did not relate to adjustable pedals and did not "necessarily go to the issue of motivation to attach the electronic control on the support bracket of the pedal assembly." *Ibid.* When the patents were interpreted in this way, the Court of Appeals held, they would not have led a person of ordinary skill to put a sensor on the sort of pedal described in Asano.

That it might have been obvious to try the combination of Asano and a sensor was likewise irrelevant, in the court's view, because " 'obvious to try" has long been held not to constitute obviousness.' " *Id.*, at 289 (quoting *In re Deuel*, 51 F.3d 1552, 1559 [34 USPQ2d 1210] (CA Fed. 1995)).

The Court of Appeals also faulted the District Court's consideration of the PTO's rejection of the broader version of claim 4. The District Court's role, the Court of Appeals explained, was not to speculate regarding what the PTO might have done had the Engelgau patent mentioned Asano. Rather, the court held, the District Court was obliged first to

presume that the issued patent was valid and then to render its own independent judgment of obviousness based on a review of the prior art. The fact that the PTO had rejected the broader version of claim 4, the Court of Appeals said, had no place in that analysis.

The Court of Appeals further held that genuine issues of material fact precluded summary judgment. Teleflex had proffered statements from one expert that claim 4 " 'was a simple, elegant, and novel combination of features,' " 119 Fed. Appx., at 290, compared to Rixon, and from another expert that claim 4 was nonobvious because, unlike in Rixon, the sensor was mounted on the support bracket rather than the pedal itself. This evidence, the court concluded, sufficed to require a trial.

II

A

[1] We begin by rejecting the rigid approach of the Court of Appeals. Throughout this Court's engagement with the question of obviousness, our cases have set forth an expansive and flexible approach inconsistent with the way the Court of Appeals applied its TSM test here. To be sure, *Graham* recognized the need for "uniformity and definiteness." 383 U.S., at 18. Yet the principles laid down in *Graham* reaffirmed the "functional approach" of *Hotchkiss*, 11 How. 248. See 383 U.S., at 12. To this end, *Graham* set forth a broad inquiry and invited courts, where appropriate, to look at any secondary considerations that would prove instructive. *Id.*, at 17.

Neither the enactment of § 103 nor the analysis in *Graham* disturbed this Court's earlier instructions concerning the need for caution in granting a patent based on the combination of elements found in the prior art. For over a half century, the Court has held that a "patent for a combination which only unites old elements with no change in their respective functions ...obviously withdraws what is already known into the field of its monopoly and diminishes the resources available to skillful men." *Great Atlantic & Pacific Tea Co.* v. *Supermarket Equipment Corp.*, 340 U.S. 147, 152 [87 USPQ 303] (1950). This is a principal reason for declining to allow patents for what is obvious. The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results. Three cases decided after *Graham* illustrate the application of this doctrine.

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In *United States* v. *Adams*, 383 U.S. 39, 40 [148 USPQ 479](1966), a companion case to *Graham*, the Court considered the obviousness of a "wet battery" that varied from prior designs in two ways: It contained water, rather than the acids conventionally employed in storage batteries; and its electrodes were magnesium and cuprous chloride, rather than zinc and silver chloride. The Court recognized that when a patent claims a structure already known in the prior art that is altered by the mere substitution of one element for another known in the field, the combination must do more than yield a predictable result. 383 U.S., at 50–51. It nevertheless rejected the Government's claim that Adams's battery was obvious. The Court relied upon the corollary principle that when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious. *Id.*, at 51–52. When Adams designed his battery, the prior art warned that risks were involved in using the types of electrodes he employed. The fact that the elements worked together in an unexpected and fruitful manner supported the conclusion that Adams's design was not obvious to those skilled in the art.

In *Anderson's-Black Rock, Inc.* v. *Pavement Salvage Co.*, 396 U.S. 57 [163 USPQ 673] (1969), the Court elaborated on this approach. The subject matter of the patent before the Court was a device combining two pre-existing elements:a radiant-heat burner and a paving machine. The device, the Court concluded, did not create some new synergy: The radiant-heat burner functioned just as a burner was expected to function; and the paving machine did the same. The two in combination did no more than they would in separate, sequential operation. *Id.*, at 60–62. In those circumstances, "while the combination of old elements performed a useful function, it added nothing to the nature and quality of the radiant-heat burner already patented,"and the patent failed under § 103. *Id.*, at 62 (footnote omitted).

Finally, in *Sakraida* v. *AG Pro, Inc.*, 425 U.S. 273 [189 USPQ 449] (1976), the Court derived from the precedents the conclusion that when a patent "simply arranges old elements with each performing the same function it had been known to perform" and yields no

more than one would expect from such an arrangement, the combination is obvious. *Id.*, at 282.

[2] The principles underlying these cases are instructive when the question is whether a patent claiming the combination of elements of prior art is obvious. When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, § 103 likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill. *Sakraida* and *Anderson's-Black Rock* are illustrative—a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions.

[3] Following these principles may be more difficult in other cases than it is here because the claimed subject matter may involve more than the simple substitution of one known element for another or the mere application of a known technique to a piece of prior art ready for the improvement. Often, it will be necessary for a court to look to interrelated teachings of multiple patents;the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue. To facilitate review, this analysis should be made explicit. See *In re Kahn*, 441 F.3d 977, 988 [78 USPQ2d 1329] (CA Fed. 2006) ("[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness"). As our precedents make clear, however, the analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ.

B

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[4] When it first established the requirement of demonstrating a teaching, suggestion, or motivation to combine known elements in order to show that the combination is obvious, the Court of Customs and Patent Appeals captured a helpful insight. See *Application of Bergel,* 292 F.2d 955, 956–957[130 USPQ 206](1961). As is clear from cases such as *Adams,* a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. Although common sense directs one to look with care at a patent application that claims as innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does. This is so because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known.

Helpful insights, however, need not become rigid and mandatory formulas; and when it is so applied, the TSM test is incompatible with our precedents. The obviousness analysis cannot be confined by a formalistic conception of the words teaching, suggestion, and motivation, or by overemphasis on the importance of published articles and the explicit content of issued patents. The diversity of inventive pursuits and of modern technology counsels against limiting the analysis in this way. In many fields it may be that there is little discussion of obvious techniques or combinations, and it often may be the case that market demand, rather than scientific literature, will drive design trends. Granting patent protection to advances that would occur in the ordinary course without real innovation retards progress and may, in the case of patents combining previously known elements, deprive prior inventions of their value or utility.

In the years since the Court of Customs and Patent Appeals set forth the essence of the TSM test, the Court of Appeals no doubt has applied the test in accord with these principles in many cases. There is no necessary inconsistency between the idea underlying the TSM test and the *Graham* analysis. But when a court transforms the general principle into a

rigid rule that limits the obviousness inquiry, as the Court of Appeals did here, it errs.

C

The flaws in the analysis of the Court of Appeals relate for the most part to the court's narrow conception of the obviousness inquiry reflected in its application of the TSM test. In determining whether the subject matter of a patent claim is obvious, neither the particular motivation nor the avowed purpose of the patentee controls. What matters is the objective reach of the claim. If the claim extends to what is obvious, it is invalid under § 103. One of the ways in which a patent's subject matter can be proved obvious is by noting that there existed at the time of invention a known problem for which there was an obvious solution encompassed by the patent's claims.

[5] The first error of the Court of Appeals in this case was to foreclose this reasoning by holding that courts and patent examiners should look only to the problem the patentee was trying to solve. 119 Fed. Appx., at 288. The Court of Appeals failed to recognize that the problem motivating the patentee may be only one of many addressed by the patent's subject matter. The question is not whether the combination was obvious to the patentee but whether the combination was obvious to a person with ordinary skill in the art. Under the correct analysis, any need or problem known in the field of endeavor at the time of invention and addressed by the patent can provide a reason for combining the elements in the manner claimed.

The second error of the Court of Appeals lay in its assumption that a person of ordinary skill attempting to solve a problem will be led only to those elements of prior art designed to solve the same problem. *Ibid.* The primary purpose of Asano was solving the constant ratio problem; so, the court concluded, an inventor considering how to put a sensor on an adjustable pedal would have no reason to consider putting it on the Asano pedal. *Ibid.* Common sense teaches, however, that familiar items may have

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obvious uses beyond their primary purposes, and in many cases a person of ordinary skill will be able to fit the teachings of multiple patents together like pieces of a puzzle. Regardless of Asano's primary purpose, the design provided an obvious example of an adjustable pedal with a fixed pivot point; and the prior art was replete with patents indicating that a fixed pivot point was an ideal mount for a sensor. The idea that a designer hoping to make an adjustable electronic pedal would ignore Asano because Asano was designed to solve the constant ratio problem makes little sense. A person of ordinary skill is also a person of ordinary creativity, not an automaton.

[6] The same constricted analysis led the Court of Appeals to conclude, in error, that a patent claim cannot be proved obvious merely by showing that the combination of elements was "obvious to try." *Id.*, at 289 (internal quotation marks omitted). When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under § 103.

The Court of Appeals, finally, drew the wrong conclusion from the risk of courts and patent examiners falling prey to hindsight bias. A factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon ex *post* reasoning. See *Graham*, 383 U.S., at 36 (warning against a "temptation to read into the prior art the teachings of the invention in issue" and instructing courts to " 'guard against slipping into the use of hindsight' "(quoting *Monroe Auto Equipment Co.* v. *Heckethorn Mfg. & Supply Co.*, 332 F.2d 406, 412[141 USPQ 549](CA6 1964))). Rigid preventative rules that deny factfinders recourse to common sense, however, are neither necessary under our case law nor consistent with it.

We note the Court of Appeals has since elaborated a broader conception of the TSM test than was applied in the instant matter. See, e.g., *DyStar Textilfarben GmbH & Co. Deutschland KG* v. *C. H. Patrick Co.*, 464 F.3d 1356, 1367 [80 USPQ2d 1641] (2006) ("Our suggestion test is in actuality quite flexible and not only permits, but *requires*, consideration of common knowledge and common sense"); *Alza Corp.* v. *Mylan Labs., Inc.*, 464 F.3d 1286, 1291 [80 USPQ2d 1001] (2006)("There is flexibility in our obviousness jurisprudence because a motivation may be found *implicitly* in the prior art. We do not

have a rigid test that requires an actual teaching to combine ..."). Those decisions, of course, are not now before us and do not correct the errors of law made by the Court of Appeals in this case. The extent to which they may describe an analysis more consistent with our earlier precedents and our decision here is a matter for the Court of Appeals to consider in its future cases. What we hold is that the fundamental misunderstandings identified above led the Court of Appeals in this case to apply a test inconsistent with our patent law decisions.

III

When we apply the standards we have explained to the instant facts, claim 4 must be found obvious. We agree with and adopt the District Court's recitation of the relevant prior art and its determination of the level of ordinary skill in the field. As did the District Court, we see little difference between the teachings of Asano and Smith and the adjustable electronic pedal disclosed in claim 4 of the Engelgau patent. A person having ordinary skill in the art could have combined Asano with a pedal position sensor in a fashion encompassed by claim 4, and would have seen the benefits of doing so.

A

Teleflex argues in passing that the Asano pedal cannot be combined with a sensor in the manner described by claim 4 because of the design of Asano's pivot mechanisms. See Brief for Respondents 48–49, and n. 17. Therefore, Teleflex reasons, even if adding a sensor to Asano was obvious, that does not establish that claim 4 encompasses obvious subject matter. This argument was not, however, raised

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before the District Court. There Teleflex was content to assert only that the problem motivating the invention claimed by the Engelgau patent would not lead to the solution of combining of Asano with a sensor. See Teleflex's Response to KSR's Motion for Summary Judgment of Invalidity in No. 02–74586 (ED Mich.), pp. 18–20, App. 144a–146a. It is also unclear whether the current argument was raised before the Court of Appeals, where Teleflex advanced the nonspecific, conclusory contention that combining Asano with a sensor would not satisfy the limitations of claim 4. See Brief for Plaintiffs-Appellants in No. 04–1152 (CA Fed.), pp. 42–44. Teleflex's own expert declarations, moreover, do not support the point Teleflex now raises. See Declaration of Clark J. Radcliffe, Ph.D., Supplemental App. 204–207; Declaration of Timothy L. Andresen, *id.,* at 208–210. The only statement in either declaration that might bear on the argument is found in the Radcliffe declaration:

> "Asano ... and Rixon ... are complex mechanical linkage-based devices that are expensive to produce and assemble and difficult to package. It is exactly these difficulties with prior art designs that [Engelgau] resolves. The use of an adjustable pedal with a single pivot reflecting pedal position combined with an electronic control mounted between the support and the adjustment assembly at that pivot was a simple, elegant, and novel combination of features in the Engelgau '565 patent." *Id.,* at 206, ¶ 16.

Read in the context of the declaration as a whole this is best interpreted to mean that Asano could not be used to solve "[t]he problem addressed by Engelgau '565[:]to provide a less expensive, more quickly assembled, and smaller package adjustable pedal assembly with electronic control." *Id.,* at 205, ¶ 10.

The District Court found that combining Asano with a pivot-mounted pedal position sensor fell within the scope of claim 4. 298 F.Supp.2d, at 592–593. Given the sigificance of that finding to the District Court's judgment, it is apparent that Teleflex would have made clearer challenges to it if it intended to preserve this claim. In light of Teleflex's failure to raise the argument in a clear fashion, and the silence of the Court of Appeals on the issue, we take the District Court's conclusion on the point to be correct.

B

[7] The District Court was correct to conclude that, as of the time Engelgau designed the subject matter in claim 4, it was obvious to a person of ordinary skill to combine Asano with a pivot-mounted pedal position sensor. There then existed a marketplace that created a strong incentive to convert mechanical pedals to electronic pedals, and the prior art taught a number of methods for achieving this advance. The Court of Appeals considered the issue too narrowly by, in effect, asking whether a pedal designer writing on a blank slate would have chosen both Asano and a

modular sensor similar to the ones used in the Chevrolet truckline and disclosed in the '068 patent. The District Court employed this narrow inquiry as well, though it reached the correct result nevertheless. The proper question to have asked was whether a pedal designer of ordinary skill, facing the wide range of needs created by developments in the field of endeavor, would have seen a benefit to upgrading Asano with a sensor.

In automotive design, as in many other fields, the interaction of multiple components means that changing one component often requires the others to be modified as well. Technological developments made it clear that engines using computer-controlled throttles would become standard. As a result, designers might have decided to design new pedals from scratch; but they also would have had reason to make pre-existing pedals work with the new engines. Indeed, upgrading its own pre-existing model led KSR to design the pedal now accused of infringing the Engelgau patent.

For a designer starting with Asano, the question was where to attach the sensor. The consequent legal question, then, is whether a pedal designer of ordinary skill starting with Asano would have found it obvious to put the sensor on a fixed pivot point. The prior art discussed above leads us to the conclusion that attaching the sensor where both KSR and Engelgau put it would have been obvious to a person of

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ordinary skill.

The '936 patent taught the utility of putting the sensor on the pedal device, not in the engine. Smith, in turn, explained to put the sensor not on the pedal's footpad but instead on its support structure. And from the known wire-chafing problems of Rixon, and Smith's teaching that "the pedal assemblies must not precipitate any motion in the connecting wires," Smith, col. 1, lines 35–37, Supplemental App. 274, the designer would know to place the sensor on a nonmoving part of the pedal structure. The most obvious nonmoving point on the structure from which a sensor can easily detect the pedal's position is a pivot point. The designer, accordingly, would follow Smith in mounting the sensor on a pivot, thereby designing an adjustable electronic pedal covered by claim 4.

Just as it was possible to begin with the objective to upgrade Asano to work with a computer-controlled throttle, so too was it possible to take an adjustable electronic pedal like Rixon and seek an improvement that would avoid the wire-chafing problem. Following similar steps to those just explained, a designer would learn from Smith to avoid sensor movement and would come, thereby, to Asano because Asano disclosed an adjustable pedal with a fixed pivot.

Teleflex indirectly argues that the prior art taught away from attaching a sensor to Asano because Asano in its view is bulky, complex, and expensive. The only evidence Teleflex marshals in support of this argument, however, is the Radcliffe declaration, which merely indicates that Asano would not have solved Engelgau's goal of making a small, simple, and inexpensive pedal. What the declaration does not indicate is that Asano was somehow so flawed that there was no reason to upgrade it, or pedals like it, to be compatible with modern engines. Indeed, Teleflex's own declarations refute this conclusion. Dr. Radcliffe states that Rixon suffered from the same bulk and complexity as did Asano. See *Id.*, at 206. Teleflex's other expert, however, explained that Rixon was itself designed by adding a sensor to a pre-existing mechanical pedal. See *Id.*, at 209. If Rixon's base pedal was not too flawed to upgrade, then Dr. Radcliffe's declaration does not show Asano was either. Teleflex may have made a plausible argument that Asano is inefficient as compared to Engelgau's preferred embodiment, but to judge Asano against Engelgau would be to engage in the very hindsight bias Teleflex rightly urges must be avoided. Accordingly, Teleflex has not shown anything in the prior art that taught away from the use of Asano.

Like the District Court, finally, we conclude Teleflex has shown no secondary factors to dislodge the determination that claim 4 is obvious. Proper application of *Graham* and our other precedents to these facts therefore leads to the conclusion that claim 4 encompassed obvious subject matter. As a result, the claim fails to meet the requirement of § 103.

We need not reach the question whether the failure to disclose Asano during the prosecution of Engelgau voids the presumption of validity given to issued patents, for claim 4 is obvious despite the presumption. We nevertheless think it appropriate to note that the rationale underlying the presumption—that the PTO, in its expertise, has approved the claim—seems much diminished here.

IV

[8] A separate ground the Court of Appeals gave for reversing the order for summary judgment was the existence of a dispute over an issue of material fact. We disagree with the Court of Appeals on this point as well. To the extent the court understood the *Graham* approach to exclude the possibility of summary judgment when an expert provides a conclusory affidavit addressing the question of obviousness, it misunderstood the role expert testimony plays in the analysis. In considering summary judgment on that question the district court can and should take into account expert testimony, which may resolve or keep open certain questions of fact. That is not the end of the issue, however. The ultimate judgment of obviousness is a legal determination. *Graham*, 383 U.S., at 17. Where, as here, the content of the prior art, the scope of the patent claim, and the level of ordinary skill in the art are not in material dispute, and

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the obviousness of the claim is apparent in light of these factors, summary judgment is appropriate. Nothing in the declarations proffered by Teleflex prevented the District Court from reaching the careful conclusions underlying its order for summary judgment in this case.

* * *

We build and create by bringing to the tangible and palpable reality around us new works based on instinct, simple logic, ordinary inferences, extraordinary ideas, and sometimes even genius. These advances, once part of our shared knowledge, define a new threshold from which innovation starts once more. And as progress beginning from higher levels of achievement is expected in the normal course, the results of ordinary innovation are not the subject of exclusive rights under the patent laws. Were it otherwise patents might stifle, rather than promote, the progress of useful arts. See U.S. Const., Art. I, § 8, cl. 8. These premises led to the bar on patents claiming obvious subject matter established in *Hotchkiss* and codified in § 103. Application of the bar must not be confined within a test or formulation too constrained to serve its purpose.

KSR provided convincing evidence that mounting a modular sensor on a fixed pivot point of the Asano pedal was a design step well within the grasp of a person of ordinary skill in the relevant art. Its arguments, and the record, demonstrate that claim 4 of the Engelgau patent is obvious. In rejecting the District Court's rulings, the Court of Appeals analyzed the issue in a narrow, rigid manner inconsistent with § 103 and our precedents. The judgment of the Court of Appeals is reversed, and the case remanded for further proceedings consistent with this opinion.

It is so ordered.

- End of Case -

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